5-2017

Thermal Inactivation of Desiccation-adapted *Salmonella enterica* in Physically Heat-treated Poultry Litter Used as Biological Soil Amendments

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THERMAL INACTIVATION OF DESICCATION-ADAPTED *SALMONELLA ENTERICA* IN PHYSICALLY HEAT-TREATED POULTRY LITTER USED AS BIOLOGICAL SOIL AMENDMENTS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

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by
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May 2017

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ABSTRACT

Poultry litter is usually recycled into the soil to improve the structure and fertility of agricultural land. As an important source of nutrients for crop production, poultry litter may possibly contain a variety of human pathogens that can threaten humans who consume the contaminated produce. The composting process can inactivate pathogens while creating a biological soil amendment beneficial for application to arable agricultural land. Some pathogens may have the potential to survive for long periods of time in raw poultry litter or its inadequately composted products after land application, and a small population of pathogenic cells may even regrow to high levels when the conditions are favorable for growth. Further thermal processing is a good choice for inactivating pathogens in poultry litter prior to land application. However, some microbial populations may become acclimatized to a hostile environment during composting or stockpiling and develop cross-protection against subsequent high temperature treatment. The objectives of this study were thus to 1) investigate the thermal inactivation of desiccation-adapted *S. enterica* in aged chicken litter, 2) study the effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *S. enterica*, 3) develop a two-step heat treatment for inactivating desiccation-adapted *S. enterica* in aged chicken litter, 4) select the indigenous indicator microorganisms for validating desiccation-adapted *S. enterica* reduction in physically heat-treated poultry litter, and 5) study the thermal resistance and gene expression of both desiccation-adapted and rehydrated *S. enterica* Typhimurium in aged broiler litter.

Thermal inactivation of desiccation-adapted *Salmonella enterica* in aged chicken litter was investigated in comparison with non-adapted cells to examine potential cross-tolerance of desiccation-adapted cells to heat treatment. A mixture of four *Salmonella* serovars was inoculated into the finished compost with 20, 30, 40, and 50% moisture contents for a 24-h desiccation adaptation. Afterwards, the compost with desiccation-adapted cells was inoculated into the aged chicken litter with the same moisture content for heat treatments at 70, 75, 80, 85 and 150°C. Recovery media were used to allow heat-injured cells to resuscitate. A 5-log reduction of the desiccation-adapted cells in aged chicken litter with 20% moisture content required >6, >6, 4~5, and 3~4 h exposure at 70, 75, 80, and 85ºC, respectively. As a comparison, a 5-log reduction of non-adapted cells in the same chicken litter was achieved within 1.5~2, 1~1.5, 0.5~1, and <0.5 h at 70, 75, 80, and 85ºC, respectively. Exposure time required to obtain a 5-log reduction in the desiccation-adapted cells gradually became shorter as temperature and moisture content were increased. At 150ºC, desiccation-adapted *Salmonella* survived for 50 min in chicken litter with 20% moisture content, whereas control cells were detectable by enrichment until only 10 min. Our results demonstrated that the thermal resistance of *Salmonella* in aged chicken litter was increased significantly when the cells were adapted to desiccation. This study also validated the effectiveness of thermal processing being used for producing chicken litter free of Salmonella contamination.

The effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *Salmonella* were evaluated. Chicken litter was kept as a stacked heap on a poultry farm and samples were collected up to 9 months of storage.

Chicken litter inoculated with desiccation-adapted *Salmonella* cells was heat-treated at 75, 80, 85, and 150ºC. *Salmonella* populations decreased in all these samples during heat treatment, and the inactivation rates became slower in chicken litter when storage time was extended from 0 to 6 months. There was no significant difference ($P>0.05$) in thermal resistance of *Salmonella* in 6- and 9-month litter samples indicating that a threshold for thermal resistance was reached after 6 months. Overall, the thermal resistance of *Salmonell*a in chicken litter was affected by the storage time of the litter. The changes in some chemical, physical, and microbiological properties during storage could possibly contribute to this difference. Moisture and ammonia could be two of the most significant factors influencing the thermal resistance of *Salmonella* cells in chicken litter. Our results emphasize the importance of adjusting time-temperature conditions for heat processing chicken litter when it is removed from the chicken house at different time intervals.

The effectiveness of a two-step heat treatment for eliminating desiccation-adapted *Salmonella* spp. in aged chicken litter was evaluated. The aged chicken litter with 20, 30, 40, and 50% moisture contents was inoculated with a mixture of 4 *Salmonella* serotypes for a 24-h adaptation. Afterwards, the inoculated chicken litter was added into the chicken litter with the adjusted moisture content for a 1-h moist-heat treatment at 65°C and 100% relative humidity inside a water bath, followed by a dry-heat treatment in a convectional oven at 85°C for 1 h to the desired moisture level (<10~12%). After moist-heat treatment, the populations of *Salmonella* in aged chicken litter at 20 and 30% moisture contents declined from ca. 6.70 log cfu/g to 3.31 and 3.00 log cfu/g,

respectively. And after subsequent 1-h dry-heat treatment, the populations further decreased to 2.97 and 2.57 log cfu/g, respectively. *Salmonella* cells in chicken litter with 40 and 50% moisture contents were only detectable by enrichment after 40 and 20 min of moist-heat treatment, respectively. Moisture contents in all samples were reduced to <10% after 1-h dry-heat process. Our results demonstrated that the two-step heat treatment was effective in reducing >5.5 logs of desiccation-adapted *Salmonella* in aged chicken litter with moisture content at or above 40%. Clearly, the findings from this study may provide chicken litter processing industry with an effective heat treatment method for producing *Salmonella*-free chicken litter.

The indigenous indicator microorganisms for validating desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter was selected. The thermal resistance of desiccation-adapted *S.* Senftenberg 775/W was compared with those of indigenous enterococci and total aerobic bacteria in poultry litter. Aged broiler litter and composted turkey litter with 20, 30, 40, and 50% moisture contents were inoculated with desiccation-adapted *S.* Senftenberg 775/W, and then heat-treated at 75 and 85ºC. Compared to total aerobic bacteria, there were better correlations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci in broiler litter samples with 20, 30, 40, and 50% moisture contents at 75ºC (R^2>0.91), and 20, 30, and 40% moisture contents at 85ºC (R^2>0.87). The mean log reductions of *S.* Senftenberg 775/W were better-correlated with those of indigenous enterococci in turkey litter samples with 20, 30, 40, and 50% moisture contents at 75ºC (R^2>0.88), and 20 and 30% moisture contents at 85ºC (R^2=0.83) than those of total aerobic bacteria, which had a

better correlation in turkey litter sample with 40% (R^2=0.98) moisture content at 85ºC. Indigenous enterococci may be used to validate the thermal processing of poultry litter as it mimics the survival behavior of *Salmonella* under some treatment conditions. This study provides some scientific data for poultry litter processors when validating the effectiveness of thermal processing.

The thermal resistance and gene expression of both desiccation-adapted and rehydrated *S.* Typhimurium in aged broiler litter was investigated. *S.* Typhimurium cells were desiccation-adapted in aged broiler litter with 20% moisture content (a_w: 0.81) for 3, 12, or 24 h at room temperature and then rehydrated for 3 h. Four genes (*rpoS*, *proV*, *dnaK*, and *grpE*) were up-regulated under desiccation stress (P<0.05) and they could be induced within an even shorter period of time (after 1 h but less than 2 h). Following rehydration, fold changes of these four genes became significantly lower (P<0.05). Desiccation-adapted Δ*rpoS Salmonella* mutant was less heat-resistant at 75°C than desiccation-adapted wild type (P<0.05), whereas there were no differences in heat resistance between desiccation-adapted *Salmonella* mutants in two non-regulated genes (*otsA* and *PagfD*) and desiccation-adapted wild type (P>0.05). Survival characteristics of desiccation-adapted *ΔPagfD* (rdar morphotype) and Δ*agfD* (saw morphotype) were similar (P>0.05). The trehalose synthesis in 3-, 12-, or 24-h desiccation-adapted wild type was not significantly induced as compared to non-adapted cells (P>0.05). Our results demonstrated the importance of *rpoS*, *proV*, *dnaK*, and *grpE* genes in the desiccation survival of *S.* Typhimurium. Moreover, *rpoS* gene was identified to be involved in the cross-protection of desiccation-adapted *S.* Typhimurium against high temperature, while

trehalose synthesis or rdar morphology did not play a significant role in this phenomenon. *S.* Typhimurium could respond rapidly to the low-a_w condition in aged broiler litter while developing the cross-protection against high temperature.

Our results suggested the thermal resistance of desiccation-adapted *Salmonella* in poultry litter could be affected by litter storage time and ammonia content. Desiccation-adapted *Salmonella* in poultry litter could produce cross-protection against subsequent thermal stress. And *rpoS* has been found to play a significant role in this cross-protection. A two-step thermal processing technique was developed to rapidly inactivate desiccation-adapted *Salmonella* in poultry litter. Indigenous enterococci can be used as an indicator microorganism to validate the thermal processing of poultry litter, as it mimics the survival behavior of desiccation-adapted *Salmonella* during heat treatment. This study provides some valuable information for poultry litter processors to control human pathogens in poultry litter as biological soil amendments.

DEDICATION

I wish to dedicate this work to my parents. This dissertation would not have been possible without their everlasting love and encouragement during the struggles of my education. Dad and mom, thank you for guiding me to become the person who I am today and instilling in me the ethic of working hard and self-motivation. I also wish to thank my wife for her endless love, patience, and support in good and in bad times. I can never thank her enough for her unwavering understanding through the ups and downs of my study, as well as for the sacrifices she made so that I could accomplish one of my biggest dreams in life. My sincere and heartfelt gratitude also extends to my relatives who always support me in all my endeavors. Finally, a special dedication to my grandfather, who always encouraged me to pursue higher education since I was a child. I believe he is now smiling down from Heaven and is very proud of all my achievements.

ACKNOWLEDGMENTS

Appreciation is expressed for the guidance of my supervisor, Dr. Xiuping Jiang under whose direction this research was developed into a doctoral dissertation. I would like to express my deepest gratitude to her for her continuous assistance, encouragement, and support throughout my Ph.D. study. My appreciation also goes to all committee members, Dr. Min Cao, Dr. Annel K. Greene, Dr. J. Michael Henson, and Dr. Tzuen-Rong Jeremy Tzeng, for their help and time during my Ph.D. pursuit. I would also like to thank my lab mates-I appreciate all of your friendship and support. You're one of a kind.

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CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

Poultry Litter

Poultry litter is a waste by-product of poultry production, which consists of bedding and feeding materials, feathers, and manure. Approximately 14 million tons of litter and manure were produced on U.S. poultry farms in 1990 and over 90% of poultry litter was applied to agricultural land (Moore et al., 1995). Poultry litter has a high nutritional value for supporting plant growth, containing essential nutrients such as nitrogen (N), phosphorus (P), and potassium (K), and also trace elements, such as copper (Cu) and zinc (Zn) (Kelleher et al., 2002). Land application offers the best solution to manage the enormous amounts of chicken litter generated on U.S. poultry farms annually. In terms of the characteristic and composition of poultry litter, it can enhance crop production by supplying various nutrients and increasing arable soil quality.

Salmonella in Poultry Litter

Poultry litter contains a large and diverse population of microorganisms. The bacterial concentration in poultry litter can reach up to 10 log cfu/g, and Gram-positive bacteria, such as actinomycetes, clostridia/eubacteria, and bacilli/lactobacilli, account for nearly 90% of the microbial diversity (Bolan et al., 2010). Pathogens in poultry litter represent the major group of bacteria of special interest to litter processors. A variety of human pathogens can be found in poultry litter, such as *Actinobacillus*, *Bordetella*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Escherichia coli*, *Globicatella*, *Listeria*,

Mycobacterium, *Salmonella*, *Staphylococcus*, and *Streptococcus* (Alexander et al., 1968; Lovett et al., 1971; Lu et al., 2003; Stern and Robach, 2003; Ngodigha et al., 2009; Bolan et al., 2010).

Active surveillance data on foodborne diseases from the United States reveal that among pathogens associated with foodborne outbreaks, *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter*, and *Listeria monocytogenes* are responsible for the majority of outbreaks (CDC, 2011). *Salmonella* spp. is the most widely distributed pathogen in poultry litter with poultry and eggs remaining as the predominant reservoir. During 1998-2008, foodborne disease outbreaks caused by *Salmonella* were associated most commonly with poultry meat products (30%) and eggs (24%) (CDC, 2013). Chicken eggs can be contaminated with *Salmonella* either horizontally or vertically. The contamination of egg shell can result from horizontal transmission, such as fecal contact (Forsythe et al., 1967). And vertical transmission of *Salmonella* has been observed in infected ovaries, oviducts, or infected eggs (Foley et al., 2011). Although only low numbers of *Salmonella* can contaminate eggs via the fecal route, these small populations cannot be ignored. Noticeably, *S*. Enteritidis, *S*. Typhimurium, or *S*. Heidelberg present in chicken feces may not only penetrate into the interior of eggs but also multiply during storage (Schoeni et al., 1995). *Salmonella* is more frequently isolated from poultry litter or fecal samples as compared to other pathogens being investigated and its prevalence level can range widely from 0 to 100%. And the population of *Salmonella* in chicken litter can range from 4 to 1.1×10^5 MPN/g litter (Chinivasagam et al., 2010). As shown in Table 1.1, microbiological surveys have revealed the prevalence of *Salmonella* in poultry litter,

Table 1.1 Prevalence of *Salmonella* in poultry litter

Year/Location	Sample type	Sample size	Prevalence	References
N.A./Canada	Poultry litter samples	44	7%	Alexander et al. (1968)
N.A./US	Poultry litter samples	198	73%-89%	Smyser and Snoeyenbos (1969)
1977/Canada	Broiler litter samples (top 1.27 to 2.54 cm layer)	N.A.[a]	0%-2%	Bhargava et al. (1983)
1978-1979/Canada	Broiler litter samples	15 from each house	30%	Long et al. (1980)
1980-1981/Canada	Broiler litter and fecal samples	36 and 2 for litter and feces samples, respectively	19%-89% and 0%-100% for feces and litter, respectively	Higgins et al. (1982)
1989-1990/Canada	Broiler litter samples	12	76%	Renwick et al. (1992)
1994-1995/US	Poultry litter samples (64 composted, 18 not composted, and no determination for 4 samples)	86	-[b]	Martin et al. (1998)
1996-1997/US	Poultry litter samples intended for dairy cattle feed from 13 dairy ranches	104	-	Jeffrey et al. (1998)
2002/Nigeria	Poultry fecal samples	120	38%	Orji et al. (2005)

2006-2007/Hungary	Broiler fecal samples	60	35%-43%	Nógrády et al. (2008)
N.A./US	Hen fecal samples	78	17%-56%	Li et al. (2007)
N.A./Nigeria	Layer litter samples	N.A.	+[c]	Ngodigha et al. (2009)
N.A./US	Broiler fecal samples	420	6%-39%	Alali et al. (2010)
N.A./Australia	Broiler litter samples	60 sites/shed and three sets of 20 were combined	71%	Chinivasagam et al. (2010)
2004-2007/US	Samples of compost heaps with chicken litter or chicken carcasses	N.A.	26% surface and 6.1% internal samples (1st composting phase); absent in all samples (2nd composting phase)	Shepherd et al. (2010)

[a] N.A., not applicable; [b] -, no pathogen or selected microorganism was isolated; [c] +, pathogen or selected microorganism was isolated.

depending on serotype, bird age, seasonality, geographic area, farm handling practice, and so on (Higgins et al., 1982; Renwick et al., 1992; Stern and Robach, 2003; Li et al., 2007). For example, Li et al. (2007) observed that fecal samples of 18-week-old layer birds had the highest prevalence of *Salmonella* (55.6%), followed by the 25- to 28-week-old birds (41.7%), 75- to 78-week-old birds (16.7%), and 66- to 74-week-old birds (5.5%). Renwick et al. (1992) surveyed randomly selected commercial broiler chicken flocks in Canada to determine flock and management factors associated with the prevalence of *Salmonella* contamination in the floor litter. They found that the prevalence of *Salmonella* in floor litter samples was significantly associated with the age of the flock and the region of Canada in which the flock was located.

The gastrointestinal tracts of animals are the natural habitats for most of the enteric pathogens. After being defecated in feces, these pathogens are immediately exposed to a hostile environment with numerous microorganisms to compete for limited nutrients. Botts et al. (1952) and Tucker (1967) found that *S*. Pullorum and *S*. Gallinarum persisted much longer in fresh chicken litter than in stockpiling litter. Other studies have also shown that some pathogens in fresh chicken litter can initially grow to higher numbers under favorable environmental conditions. Himathongkham and Riemann (1999) reported that *E*. *coli* O157:H7 and *L*. *monocytogenes* could multiply by as much as 100 folds for a period of 2 days in fresh chicken manure at 20 °C, whereas *S*. Typhimurium populations remained stable. Therefore, special attention should be paid to the initial disinfection processing so as to effectively eliminate pathogens from poultry litter.

When poultry litter as biological soil amendments are introduced into the agricultural field, the antagonistic effect of indigenous soil microorganisms and the hostile condition of soil microcosm are possible factors influencing the length of time that human pathogens can persist (Fenlon et al., 2000). According to the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption proposed in the U.S. Food and Drug Administration (USFDA) Food Safety Modernization Act (FSMA) (USFDA, 2015), growth of human pathogens in biological soil amendments of animal origin could result in the amendments acting as an inoculum that spreads pathogens to covered produce growing area, leading to a likelihood of produce contamination. Previous studies have reported the growth and persistence of human pathogens in chicken manure and manure-amended soil. Islam et al. (2004) reported that *S.* Typhimurium persisted for 203 to 231 days in soils amended with poultry compost, dairy compost, and alkaline-pH-stabilized dairy compost.

Physical Heat Treatments of Animal Wastes

To prevent foodborne disease outbreaks and to recycle huge amounts of organic wastes into an important source of organic fertilizer for agricultural use, animal wastes, such as poultry litter, should be treated to reduce or eliminate human pathogens. One of the most common types of animal waste treatments is composting. This biological process is driven by a variety of microorganisms and generates heat that can inactivate pathogens in animal wastes while creating biological soil amendments beneficial for arable land (Berry et al., 2013). Properly composted animal wastes are considered a high-quality and safe biological soil amendment, but improper composting (e.g., heating up

too slowly, not turning piles or not using insulating covers to avoid disparities in temperature stratification) may lead to the survival of pathogens that may contaminate fresh produce when the compost is applied to agricultural land for growing vegetables (Erickson et al., 2014). As a result, pathogens originally present in animal wastes may persist in the finished compost products for long periods of time (Erickson et al., 2010; Macklin et al., 2008). And a very low residual population of bacterial pathogens present in the original materials may regrow during the curing stage of the composting process (Kim et al., 2009). Hence, alternative appropriate processing and control strategies should be developed to inactivate human pathogens in animal wastes.

Physical heat treatments, such as pelletization, pasteurization, and dry heating at higher temperatures (60 to 95°C), are highly recommended to eliminate pathogens potentially present in animal wastes while drying the materials to the moisture content of <10 to 12% (Cox et al., 1986; López-Mosquera et al., 2008). Physically heat-treated animal wastes have gradually gained popularity as biological soil amendments for both organic and conventional farms (Turner et al., 1997). However, in 2006, during investigation of the *E. coli* O157:H7 outbreak associated with Dole prepackaged spinach from True Organic Products Inc. (California Food Emergency Response Team, 2007), chicken litter pellets that had been produced by exposing the composted chicken litter mixed with feather meal at 82 to 93°C to heated air for approximately 30 min were identified as a potential source of contamination. Unfortunately, there have been few studies to validate whether the heat treatment conditions during animal waste pelletizing are sufficient to inactivate human pathogens.

Table 1.2 presents the time-temperature requirements and acceptance criteria for physical heat treatments of animal manure or biosolids as biological soil amendments. Although a number of organizations or federal agencies offer independent protocol to ensure effective heat treatment for animal wastes, there are still no defined heat sources (dry *vs*. moist heat), time-temperature requirement, and microbial standards.

Laboratory-based studies. Table 1.3 presents some studies on lab-scale physical heat treatments of animal wastes for microbial inactivation, indicating that time-temperature combination requirements for eliminating pathogens differ among studies. Various animal wastes have been studied, such as dairy manure, poultry litter, and pig slurry. Different target microorganisms have also been investigated in these studies, including indigenous microflora or indicator microorganism (e.g. coliforms, total aerobic bacteria), animal virus (e.g., swine vesicular disease virus [SVDV], African swine fever virus [ASFV]), and artificially spiked human pathogen (e.g., *E. coli* O157:H7, *Salmonella* spp.) or parasite egg (e.g., *Ascaris suum*, *Ascaridia galli*). Initial levels of tested microorganisms ranged from 2 to 10 log cfu or pfu/g or ml. Studies on the thermal inactivation of various pathogens in animal wastes have yielded generally satisfactory results. However, several factors affect microbial inactivation in animal wastes during physical heat treatments. Bacteria, viruses, and parasite eggs were reported to be inactivated at 40 to 121°C in 5 min to 24 h, at 40 to 70°C in 2 min to 24 h, and at 40 to 90°C in 30 to 36 h, respectively, depending on experimental design, treatment condition, and target microorganism. Generally, microorganisms can be killed more rapidly at higher temperatures (e.g., within 1 h at 121°C) (Caswell et al., 1975). According to the

Table 1.2 Time-temperature requirements and acceptance criteria for physical heat treatments of animal wastes as biological soil amendments

Source	Biological soil amendment	Time-temperature requirement	Acceptance criterion	
			Moisture level	Microbial level
USEPA (2003)	Biosolids	Either the temperature of the biosolids >80 °C or the wet bulb temperature of the gas in contact with the biosolids as the biosolids leave the dryer >80 °C	<10%	For Class A biosolids, fecal coliforms: <1000 MPN/g dry weight or *Salmonella*: <3 MPN/4 g dry weight
National Organic Program (2006)	Animal manure	>65 °C for >60 min	<12%	Fecal coliforms, *Salmonella*, and *E. coli* O157:H7: negative
European Union (2006)	Animal manure	>70 °C for >60 min	N.A.	*E. coli* or *Enterococaceae*: <1000 MPN/g *Salmonella*: absence in 25 g of sample
California Leafy Green Products Handler Marketing Agreement (2010)	Animal manure	Either the process has been validated by a recognized authority or is subject to 150 °C for 60 min	<30%	Fecal coliforms, *Salmonella*, and *E. coli* O157:H7: negative or less than detection limit

[a] N.A., not applicable.

studies summarized in Table 1.3, overall, animal viruses were relatively heat sensitive, because they can be inactivated at lower temperatures in shorter time periods compared to bacteria or parasite eggs. However, comparisons among these different studies should be made with some precautions due to differences in the aforementioned factors and in heat source (e.g., oven, water bath, or autoclave) as well. Although Morishita and Miyaki (1979) found indigenous spore-forming *Bacillus* to be the principal components of the heat-resistant microflora of heat-treated rat feces, there is no available information about the survival of heat-resistant spore-forming human pathogens in animal wastes during lab-scale physical heat treatments. As reported by Turner and Williams (1999), inactivation of SVDV and ASFV in pig slurry depended on the slurry characteristics, such as chemical oxygen demand, total solids, and nitrogen content. In some studies, pretreatments such as urea addition, Co^{60} treatment, and anaerobic digestion were introduced before subsequent physical heat treatment (Collins et al., 2013; Katakam et al., 2014; Messer et al., 1971). For example, Katakam et al. (2014) demonstrated that urea addition increased aqueous ammonia concentrations and, thereby, markedly expedited the thermal inactivation of parasite eggs due to hurdle effect. Most of these lab-scale studies have shown a complete inactivation of the target microorganisms under certain treatment conditions; however, the experiments were only performed under lab conditions, which could differ from real-world industrial settings.

Pilot-scale studies. Studies on pilot-scale physical heat treatments of animal wastes for microbial inactivation have been reported (Table 1.4), with the processing capacity ranging from 50 to 220 liters/h. In an early work, Turner et al. (1998) designed a pilot-

scale heat treatment system with a processing capacity of 20 to 100 liters/h, which processed pig slurry at 65°C and maintained this temperature for a minimum of 5 min to provide a reasonable safety margin for viral inactivation. Based on the results, they proposed that the effectiveness of a particular treatment should be demonstrated and that the minimum level of treatment necessary to achieve a particular reduction should also be determined. Their design thus avoided over-engineering (an excessively complicated process) and identified suitable safety margins. And they also demonstrated that the system can be effective in inactivating various animal viruses in pig slurry.

Basically, pilot-scale physical heat treatments of animal wastes can involve applying heat through different means; however, most of the pilot systems in the published studies were based on the technique developed by Turner et al. (1998), as aforementioned. Also note that these pilot-scale studies only used pig slurry as the animal waste matrix. In all these pilot studies, microorganisms were reduced or eliminated with different inactivation rates, depending on time-temperature combination and tested microorganism. Most of the treatments are temperature-dependent, and microbial inactivation is usually enhanced at higher temperatures, accompanied by extended treatment times. Animal wastes require contact with heat at any given temperature for a minimum time period, indicating that the heating process in a pilot-scale dryer should operate over a sufficient time period to ensure that all the materials reach this required contact time.

Table 1.3 Lab-scale physical heat treatments of animal wastes for microbial inactivation

Animal wastes	Tested microorganisms	Initial populations	Treatment conditions	Significant results	Reference
Woodchip poultry litter, corncob poultry litter, Co^{60} woodchip poultry litter, and Co^{60} corncob poultry litter	*E. coli*, *S.* Pullorum, and *Arizona sp.*	4-5 log cfu/g	Placed in a thermal death tube (13×17 mm) and heated in a water bath at 57.2, 62.8, 68.3, and 73.8°C for 30 and 60 min	Woodchip poultry litter with 18% moisture content: *E. coli* inactivated at 68.3°C after 30 min Corncob poultry litter with 17% moisture content: *S.* Pullorum inactivated at 62.8°C for 30 min and *Arizona sp.* inactivated at 57.2°C after 30 min Woodchip poultry litter with 39% moisture content: *E. coli* inactivated at 57.2°C after 30 min Co^{60} woodchip poultry litter with 29% moisture content and Co^{60} corncob poultry litter with 17% moisture content: *E. coli* inactivated at 68.3°C after 30 min	Messer et al., 1971
Broiler litter	Indigenous coliforms and total aerobic bacteria	>4.5 log cfu/g	Heated in an oven or autoclave at 150°C in the oven for 10, 15, and 20 min at litter depth of 0.6 cm; at 121°C and steam pressure of 1.05 kg/cm^2 in the autoclave for 5, 10, 15, and 30 min at	Coliforms: inactivated under all conditions Total aerobic bacteria: reduced to an acceptable count at 150°C in the oven after >20 min at litter depth of 0.6 cm, at 121°C in the autoclave for 10, 15, and 30 min at litter depth of 5.0 cm, and at 150°C in the oven for 15 min at	Caswell et al., 1975

			litter depth of 5.0 cm; at 150°C in the oven for 15 min at litter depth of 0.6 and 2.5 cm immediately following the addition of 0, 1, 2, and 4 g of paraformaldehyde/100 g of litter	litter depth of 0.6 and 2.5 cm immediately following the addition of 1, 2, and 4 g of paraformaldehyde/100 g of litter	
Chicken manure	AIV[a] (H_5N_1, Thai field strain)	Not described	Placed in a small plastic Petri dish (i.d., 3.5 cm) and heated in an oven at 40°C for 120 min	Inactivated at 40°C in 15 min	Chumpolbanchorn et al., 2006
Broiler litter with pine shavings	*S.* Typhimurium	5 log cfu/g	Placed in a perforated nursery flat (41.91 × 33.0 cm) and heated in a soil steamer cart for 5, 30, and 120 min	Inactivated using steam in 30 or 120 min	Stringfellow et al., 2010
Broiler litter	*E. coli* and *S.* Typhimurium	8-9 log cfu/g	Placed in a 50-ml plastic tube in a 100-mm diameter plastic container and heated in a water bath at 45, 55, and 65°C for 24 h, and at 30, 50, and 65% moisture contents	>99% inactivation at 55 or 65°C in 1 h Moisture content had little effect on inactivation	Wilkinson et al., 2011

Aged broiler litter and fresh layer litter	*S.* Enteritidis, *S.* Heidelberg, and *S.* Typhimurium	7 log cfu/g	Placed in an aluminum pan (i.d., 10 cm) and heated in an oven at 75, 80, and 85°C for 5 h, and at 30, 40, and 50% moisture contents	Aged boiler litter with 30% moisture content: inactivated at 70, 75, or 80°C after 300, 165, and 75 min, respectively Fresh layer litter with 30% moisture content: inactivated at 70, 75, or 80°C after 105, 90, and 60 min, respectively	Kim et al., 2012
Poultry sludge	Fecal coliforms, *Salmonella* spp., and *Ascaris suum* eggs	10.5 log MPN/g of total solids, 4.5 log MPN/g of total solids, and 2 log eggs/l, respectively	Heated in a 1.5-l hermetically closed thermal reactor at 40, 50, 55, 60, 65, 70, 75, 80, and 90°C for 30, 60, 90, and 120 min	Fecal coliforms and *Salmonella* spp.: inactivated at 70°C *A. suum* eggs: inactivated at 80°C after 120 min	Ruiz-Espinoza et al., 2012
Layer manure	Total aerobic bacteria, yeast and mold, indigenous *E. coli*, and *Salmonella*	9.7, 3.4, and 7.4 log cfu/g, respectively	Placed in a drying tray (1, 2, or 3 cm×10 cm×10 cm) and heated in an oven at 40, 50, and 60°C	Total aerobic bacteria: 65.6-99.8% inactivation Yeast and mold: 74.1-99.6% inactivation *E. coli*: 99.97% inactivation *Salmonella*: 100% inactivation	Ghaly and Alhattab, 2013
Pig slurry	SVDV[b]	7-8 log pfu/ml	At 48, 52, 56, 60, and 64°C for 2, 10, 30, 60, and 120 min	Inactivated at 64°C after 2 min	Herniman et al., 1973

Pig slurry	ASFV[c] and SVDV	5.3 log HAD_{50}/ml and 7 log pfu/ml, respectively	Placed in a 25-ml glass bottle and heated in a water bath at 40, 50, and 60°C for 24 h	Inactivated at 60°C after 15 min and 50 and 60°C after 1 h, respectively	Turner et al., 1998
Pig slurry collected from two different sources	ASFV and SVDV	5.3 log HAD_{50}/ml and 7.7 log pfu/ml, respectively	Experimental setup same as Turner et al. (49) At 56, 60, and 65°C for 24 h	Inactivated at 65°C in 1 min or 65°C after 2 min, respectively Inactivation depended on the slurry characteristics	Turner and Williams, 1999
Pig slurry	FMDV, ADV, and CSFV[d]	3.8->6.0, 5.2-7.2, and ≤1.8-6.8 log pfu/ml, respectively	Experimental setup same as Turner et al. (49) FMDV: at 55, 60, 65, 67, and 70°C for 10 min ADV: at 55, 60, 62, and 65°C for 15 min CSFV: at 55, 60, 65, and 70°C for 5 min	Inactivated at 67°C after 3 min, 62°C after 3 min, and 60°C after 3 min, respectively	Turner et al., 2000a
Pig slurry	ASFV, SVDV, FMDV, and ADV	Not described	Experimental setup same as Turner et al. (49) At 55, 56, 60, 62, 65, 67, and 70°C	Inactivated at 65°C after 1 min, 65°C after 2-5 min, 70°C after 1 min, and 65°C after 1 min, respectively	Turner et al., 2000b

Pig slurry treated by urea or not treated by urea	*A. suum* and *Ascaridia galli* eggs	2 log eggs/g	Placed in a nylon bag in a 200-ml plastic bottle and heated in an incubator at 40°C for 36 h or in a water bath at 50°C for 2 h	Pig slurry treated by urea: inactivated at 40°C after 36 h, and 99% and 100% inactivation of *A. suum* and *A. galli* eggs at 50°C after 2 h, respectively Pig slurry not treated by urea: 4% and 94% inactivation of *A. suum* and *A. galli* eggs at 40°C after 36 h, , respectively, and 57% and 99% inactivation of *A. suum* and *A. galli* eggs at 50°C after 2 h, respectively	Katakam et al., 2014
Dairy manure	BEV and BPV[e]	Not described	Placed in a 1-l flask and heated in a water bath at 70°C for 5 and 30 min	Bovine enterovirus: inactivated at 70°C after 30 min Bovine parvovirus: not inactivated at 70°C after 5 or 30 min	Monteith et al., 1986
Anaerobically digested dairy manure	Indigenous *E. coli*, *Salmonella*, and fecal coliforms	3.9, 3.8, and 2.2 log MPN/g, respectively	Placed in a test tube and heated in a digestion unit with an aluminum block at 70, 75, and 80°C for 0, 15, 30, 45, 60, 75, 90, 105, and 120 min	Fecal coliforms: 85 to 95% inactivation *E. coli*: 87 to 96% inactivation *Salmonella*: 100% inactivation	Collins et al., 2013
Dairy manure	*S.* Senftenberg, *Enterococcus faecalis*, bacteriophage φX174, and porcine parvovirus	6-7 log cfu/g, 6-7 log cfu/g, 7-8 log pfu/g, and 7.2 log $TCID_{50}$/g, respectively	Placed in a tube and heated in a block thermostat at 46.0, 47.5, and 49.0°C for 24 h and at 50.5, 52, 53.5, and 55.0°C for 8 h for *S.*	*D*-values ranged 0.37 h at 55°C to 22.52 h at 46.0°C and 0.45 h at 55.0°C to 14.50 h at 47.5°C for *S.* Senftenberg and *E. faecalis*, respectively	Elving et al., 2014

			Senftenberg and *Enterococcus faecalis*; at 46.0, 47.5, 49.0, 50.5, 52.0, 53.5, and 55.0°C for 24 h and at 70°C for 1 h for Bacteriophage φX174; at 46.0, 47.5, 49.0, 50.5, 52.0, 53.5, and 55.0°C for 24 h and at 70°C for 2 h for Porcine parvovirus	*D*-values ranged from 1.51 h at 55°C to 16.46 h at 46°C and 16.77 h at 52°C to 16.99 h at 49°C for bacteriophage φX174 and porcine parvovirus, respectively	
Ovine blood, an ovine waste mixture comprising of blood, floor washings, and gut contents, and a mixture of sewage sludge, bovine blood, slurry, and creamery waste	*E. coli* O157:H7	6.5-7.0 log cfu/ml	Placed in a sterile microcentrifuge tube and heated in a water bath at 50, 60, and 72°C for 10 min	Inactivated at 60°C after 10 min	Avery et al., 2009
Animal byproducts Category 3 (hatchery waste, slaughterhouse waste, and manure)	PPV, AIV, BPV-3, FCoV, FCV[f], SVDV, *Enterobacteria* phage MS2, *Enterobacteria* phage ϕX174, and *S.* Typhimurium phage 28B	5-7 log pfu/g or log $TCID_{50}$/g	Heated in a water bath at 55 and 70°C for 1 h	Not inactivated at 70°C in 1 h	Emmoth, 2010

Rat feces	Indigenous microorganisms	7-10 log cfu/g	Placed in a 9-ml butyl rubber-stoppered tube filled with CO_2 and heated in a water bath at 80°C for 10 min	Anaerobic bacteria and the genus *Bacillus* were principal the components of the heat resistant microflora Anaerobic gram-variable cocci and straight rods were sometimes isolated	Morishita and Miyaki, 1979

[a] AIV, avian influenza virus.
[b] SVDV, swine vesicular disease virus.
[c] ASFV, African swine fever virus.
[d] FMDV, foot and mouth disease virus; ADV, Aujeszky's disease virus; CSFV, classical swine fever virus.
[e] BEV, bovine enterovirus; BPV, bovine parvovirus.
[f] PPV, porcine parvovirus; AIV, avian influenza virus; BPV-3, bovine parainfluenza virus 3; FCoV, feline coronavirus; FCV, feline calicivirus.

Table 1.4 Pilot-scale physical heat treatments of animal wastes for microbial inactivation

Animal wastes	Tested microorganisms	Initial populations	Treatment conditions	Significant results	Reference
Pig slurry	ASFV and SVDV[a]	Not described	Heated in a heat exchanger and passed through a reactor (50 l) at >65°C for >5 min	Inactivated at 56 and 60°C in 90 sec, respectively	Turner et al., 1998
Pig slurry	ASFV and SVDV	Not described	Experimental setup same as Turner et al. (49) ASFV: at 52.2°C for >5 min SVDV: at 40.7-60.1°C for >5 min	All inactivated at 52.2°C and pH 8 SVDV inactivated at 50-55°C and pH 7.5-8, and at 55-60°C and pH 6.4	Turner et al., 1999
Pig slurry	FMDV, ADV, and CSFV[b]	Not described	Experimental setup same as Turner et al. (49) FMDV: at 60.3-61.6°C and 64.3-65.4°C for >5 min ADV: at 56.0-57.8°C and 60.8-62.8°C for >5 min CSFV: at 49.0-50.5°C and 55.5-55.8 for >5 min	FMDV, ADV, and CSFV inactivated at 61, 61, and 50°C, respectively	Turner et al., 2000a
Pig slurry	ASFV, SVDV, FMDV, and ADV	Not described	Experimental setup same as Turner et al. (49) ASFV: 53.7-54.3°C for >5 min SVDV: 54.2-55.0°C for >5 min FMDV: 60.3-61.6°C for >5 min ADV: 56.0-57.8°C for >5 min	All inactivated under all treatment conditions	Turner et al., 2000b

Pig slurry	*Cryptosporidium* oocysts and *Giardia* cysts	4-5 log oocysts/l and 3 log cysts/l, respectively	Heated in a rotary trommel from 200°C at the beginning to 60°C at the end using the excess heat from the gas engines which produced electricity and heat from natural gas	4.3 log oocysts/l reduction of *Cryptosporidium* oocysts *Giardia* cysts inactivated	Reinoso and Becares, 2008
Pig slurry	Indigenous coliforms at 30°C, *E. coli*, enterococci, aero/anaerobic bacteria, F+ specific phages, and somatic phages (Morphology 1 and 2)	5.2, 4.9, 5.2, 7.6, 4.2, and 5.3 log cfu or pfu/ml, respectively	Heated in a two-part heat exchanger (length, 3 m; i.d., 9.7 m) at 78°C over 75 sec, passed through a retention unit at >76.3°C for 10 min, and then cooled to 41°C over 37 sec (developed based on Turner et al., 1998)	>4 log cfu/g reduction of coliforms, *E. coli*, and enterococci, >5 log pfu/g reduction of F+ specific phages and morphology 1, and >2 log pfu/g reduction of morphology 2 at 76°C but no >2 log cfu/g reduction of aero/anaerobic flora	Cunault et al., 2010
Pig slurry	Indigenous *E. coli*, *Salmonella*, enteroccoci, *C. perfringens*, and total culturable bacteria	Not described	Experimental setup same as Cunault et al. (14) Three steps: heated to high temperature over 75 sec, maintained at 70, 80, and 96°C for 10 min, and then cooled to low temperature over 37 sec	Vegetative forms inactivated at 80°C for 10 min but not the related spores <2 log cfu/g reduction of spore forms of total culturable bacteria and 4 log cfu/g reduction of *C. perfringens* at 96°C for 10 min	Pourcher et al., 2011
Pig slurry	Indigenous *E. coli*, enterococci, sulfite-reducing *Clostridia*, mesophilic culturable bacteria,	6-8, 6-8, 6-8, 8-9, 6-8, and 2-6 log cfu or pfu/100 ml, respectively	Experimental setup same as Cunault et al. (14) Three steps: heated to 56.1-100.3°C over 75 sec, maintained at 55.1-	4-5 log cfu/g reduction of vegetative forms at 70°C for 10 min and 60°C for 1 h but little effect on somatic	Cunault et al., 2011

	F+ specific phages, and somatic phages		96.4°C for 10 min, and then cooled to 33.1-42.3°C over 37 sec	phages or on the spore-formers, dominated by *Clostridium* spp. 3.1 log cfu/g reduction of sulfite-reducing *Clostridia;* 1.4 log cfu/g reduction of mesophilic culturable at 96°C but somatic phages were still detected. *C. botulinum* was identified among the thermotolerant mesophilic culturable bacteria	
Pig slurry	Indigenous *E. coli*, enterococci, sulfite-reducing *Clostridia*, F+ specific, and somatic phages	6.4, 7.0, 7.0, 7.5, and 5.8 log cfu or pfu/ml, respectively	Experimental setup same as Cunault et al. (14) Three steps: heated to high temperature over 75 sec, maintained at 55-96°C for 10 min, and then cooled to low temperature over 37 sec	4-5 log cfu/g reduction of vegetative forms at 70°C for 10 min but little effect on somatic phages or on the spore-formers, dominated by *Clostridium* spp. 3.1 log cfu/g reduction of sulfite-reducing *Clostridia* at 96°C but somatic phages were still detected Vegetative forms of mesophilic pathogenic bacteria inactivated at 60°C for 1 h or at 70°C for 10 min	Cunault et al., 2012
Type I and II deposits of pig slurry heated in	Indigenous mesophilic culturable bacteria	Not described	Experimental setup same as Cunault et al. (14)	The concentrations of spores alone, and of both spores and	Cunault et al., 2013

a tubular heat exchanger	Three steps: heated to high temperature over 75 sec, maintained at 55 and 80°C for 10 min, and then cooled to low temperature over 37 sec	vegetative forms in Type I fouling were around 7.4 log cfu/g The concentration of mesophilic cultivable bacteria was 8.6 log cfu/g in Type II fouling, whereas the concentration of spores was similar in the two types

[a] SVDV, swine vesicular disease virus; ASFV, African swine fever virus.
[b] FMDV, foot and mouth disease virus; ADV, Aujeszky's disease virus; CSFV, classical swine fever virus.

One way to evaluate the effectiveness of a pilot-scale physical heat treatment of animal wastes is to investigate the survival behaviors of indigenous microflora during the treatment. In some pilot-scale studies (Cunault et al., 2010; Cunault et al., 2012; Cunault et al., 2011; Pourcher et al., 2011), indicator microorganisms naturally occurring in animal wastes were thus used in consideration of the large variability in heat resistance. In the work by Cunault et al. (2011), three types of indigenous bacteria in pig slurry were chosen to cover a range of thermal resistance, including *E. coli* (the most sensitive), enterococci (intermediate), and spore-forming sulfite-reducing *Clostridia* (the most resistant). Mesophiles were also included to monitor more generally the effect of thermal treatment on a broad range of bacteria. And two indigenous viruses were also selected, including heat-resistant F^+ specific RNA coliphage and somatic coliphage. Bacterial spores and spore-forming *Clostridium* spp. in pig slurry have been found to be more heat-resistant, as reported by Pourcher et al. (2011) and Cunault et al. (2011; 2012).

Interestingly, the results on the influence of higher moisture contents of animal wastes on microbial inactivation during lab- and pilot-scale physical heat treatments vary from little (or positive) effect to negative effect. Under lab conditions, most of the published studies observed the inactivation effect being enhanced with the increase in moisture content of animal wastes (Kim et al., 2012), whereas Wilkinson et al. (2011) found that moisture content of poultry litter had little effect on inactivation of *E. coli* and *S.* Typhimurium. A different effect of moisture content on viral inactivation in pig slurry has been reported, based on some pilot-scale studies. Pig slurry generally contains more water, and the moisture content can even reach 97% (Imbeah, 1998). However, as stated

by Turner and Burton (1997), the high solid content in pig slurry expedited thermal inactivation of viruses because heat retention may be improved by the presence of solids. There is still a lack of thorough studies on the survival behaviors of bacterial pathogens in a wide range of animal wastes (solid versus liquid) processed by physical heat treatments.

When scaling up a physical heat treatment to a pilot plant, the most effective approach to reduce uncertainty is to perform the lab-scale research on actual animal wastes. However, an obvious difference exists between the almost ideal conditions that can be achieved in a controlled lab environment and the process in a pilot plant (Gertenbach and Cooper, 2009). Control of heat transfer and material flow of animal wastes at a larger scale is much more difficult to manipulate than that at lab scale, which requires specifically designed engineered systems to accomplish a uniform heating to ensure pathogen inactivation in all parts of the mass. Animal waste handling can also be a significant challenge if the heating process requires a particular particle size, bulk density, or moisture content of end products. Thus, moving from lab to pilot introduces more factors that should be considered when validating the treatment and setting treatment recommendations.

As recommended by the FSMA (USFDA, 2015), animal wastes, such as poultry litter, may be physically heat treated to create a dried, pelleted material that is low in microbial populations due to the high heat used during production. However, if the heat treatment is not uniform, the end product may still harbor human pathogens, leading to the potential contamination of the harvestable or harvested part of the crop to which it is

applied. Therefore, before broad commercial application of a certain physical heat treatment technique, its effectiveness against heat-resistant pathogens should be thoroughly assessed.

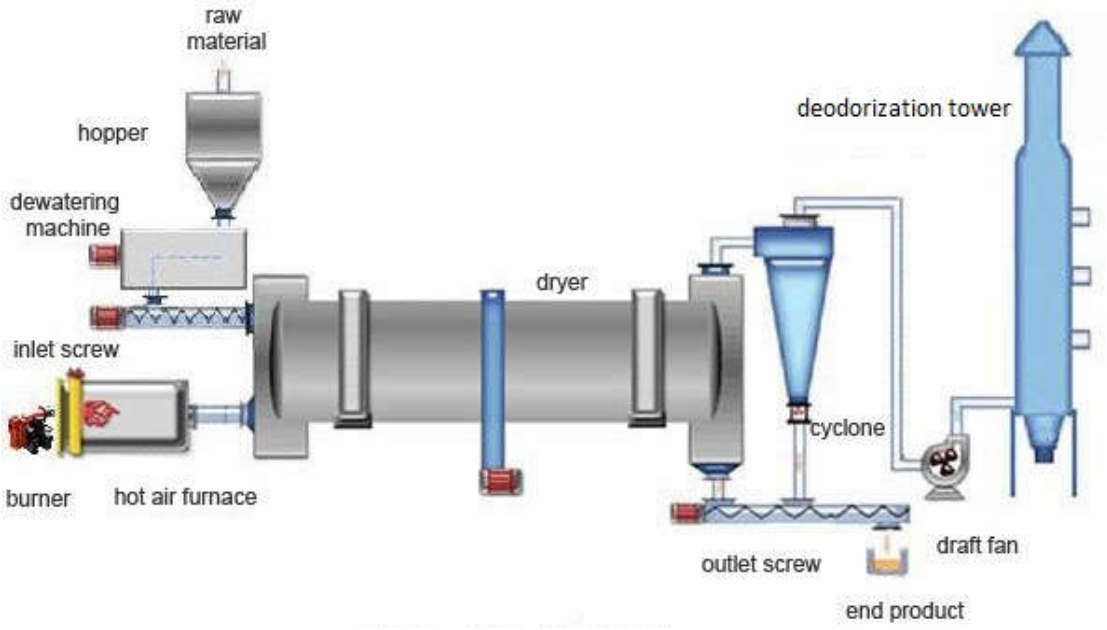
Perhaps more importantly, physically heat-treated animal wastes (e.g., heat-treated poultry manure pellets) would be expected to have relatively weak competitive microflora communities against human pathogens, resulting in the possible regrowth of pathogens in treated animal wastes (Kim et al., 2009; USFDA, 2015). Cunault et al. (2011) observed no regrowth of *E. coli* in pig slurry samples stored at 37 or 18°C for 2 weeks following pilot-scale physical heat treatment at 60°C for 10 min. Because there may be an elevated risk of regrowth of pathogenic bacteria after incompletely treated manure is spread on cropland, Cunault et al. (2011) suggested that the potential regrowth of bacteria in physically heat-treated manure should be carefully considered. Moreover, note also that pathogens introduced to physically heat-treated animal wastes through recontamination would also be more likely to grow, given the reduced numbers of competitive microflora. Hence, further comprehensive study is warranted to analyze the difference in the indigenous microbial communities in animal wastes after physical heat treatments.

Animal waste treatments at high temperatures can inactivate pathogens more rapidly, but they may also reduce the total nitrogen level and other heat-sensitive crop nutrients in the finished products (Chen and Jiang, 2014). Moreover, high temperatures can kill beneficial microorganisms that have the potential to enhance plant productivity and yield in cropping systems. Therefore, animal wastes that are physically heat treated at

high temperatures are not as microbiologically active as the composted products. Treatment conditions of low temperature over a long time and high temperature over a short time should be optimized to avoid the significant loss in nutrients and beneficial microflora during physical heat treatment.

Dry heat treatment vs. moist heat treatment. In general, dry heat takes a long time to inactivate bacterial cells in poultry litter, suggesting that current thermal processing techniques (Figure 1.1) may not rapidly eliminate pathogens from physically heat-treated poultry litter. And these surviving pathogenic cells could potentially contaminate produce and environment, when physically heat-treated poultry litter is applied to agricultural land as biological soil amendments.

Figure 1.1 Thermal processing line of poultry litter (Adapted from http://www.fte-china.com/pro/chicken_manure_dryer.html)



Furthermore, prolonged thermal exposure may utilize more energy and also negatively affect the quality of poultry litter, since mechanical drying can potentially cause nutrient loss, such as nitrogen (N) loss from ammonia volatilization (Moore et al., 1995). To achieve a rapid destruction of pathogens in physically heat-treated poultry litter while minimizing quality loss, additional approaches should be explored as hurdles for pathogen control. It is generally recognized that moist heat is a more efficient lethal treatment for microorganisms as compared to dry heat (Willey, 2008). When moist air is used to inactivate bacterial cells, much lower temperatures are required for bacterial inactivation, compared with heating with dry air. Moist heat kills microorganisms by employing water molecules to degrade nucleic acids, denature enzymes and other proteins, and disrupt cell membranes, as compared with dehydration and oxidation effects of dry heat. In animal feed industry, steam is used to condition the feed mash for rapid pathogen inactivation prior to pelletizing process (Jones, 2011). However, the response of human pathogens in animal wastes to moist heat treatment has not been thoroughly studied.

Indicator of *Salmonella* During Poultry Litter Thermal Processing

To understand the survival of pathogens in processing environment, surrogate or indicator microorganism with characteristics and behaviors similar to comparable specific pathogens are needed to perform research to avoid introducing pathogenic organisms into the environment (Harris et al., 2013). *Enterococcus faecium* NRRL B-2354 has been used as a surrogate of *Salmonella* for validating thermal processing of almonds, carbohydrate-protein meal, and so on (Jeong et al., 2011; Bianchini et al.,

2014). Almond Board of California (2007) recommended this *E. faecium* strain as a surrogate for *S.* Enteritidis PT30 in lethality studies of *Salmonella* during moist-air heating of almonds. Other studies have also demonstrated *E. faecium* as a good surrogate to study the thermal inactivation of pathogens (Annous and Kozempel, 1998; Piyasena et al., 2003). For biosolids, the enterococci indicator is a more representative surrogate of both bacterial and viral pathogen inactivation than fecal coliforms (Viau and Peccia, 2009). Only a few studies reported the evaluation of nonpathogenic microorganisms as surrogates for *Salmonella* in animal manure and compost. Two *S.* Typhimurium strains, 8243 and λ3985 Δcrp-11 Δcya-12, were used as surrogate strains for evaluating the survival of *Salmonella* during on-farm composting of dairy manure and chicken litter, respectively (Islam et al., 2004; Erickson et al., 2010; Shepherd et al., 2010). Two attenuated strains of *S.* Typhimurium, MAE 110 and MAE 119, were used to study the effects of cattle feeding regimen and soil management type on the fate of the pathogen, whereas another attenuated *S.* Typhimurium strain, PTVS177, was used to assess the root uptake and systemic vine-transport of the microorganism by melon during field study (Franz et al., 2005; Lopez-Velasco et al., 2012).

Another possible alternative for estimating pathogen survival in a processing environment is to monitor the behavior of indigenous microflora as indicator microorganisms. It is well-recognized that enterococci are very hardy microorganisms which are resistant to various stresses, such as heat and desiccation. As described above, there is high correlation of thermal resistance between enterococci and *Salmonella*. Juneja et al. (1997) reported that the spoilage microflora in ground beef patties cooked in

a skillet died almost in the same rate as the *E. coli* O157:H7. Similarly, Juneja (2003) compared the thermal inactivation of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* with that of indigenous microflora in ground beef. And they found that the slope of the thermal death time curve of indigenous microflora was the same as that of these pathogens, suggesting that it is feasible to develop a thermal process to validate the safety of a cooking approach without introducing any pathogens into a processing environment. Although studies have been reported using indicator microorganisms for food processing, there is still no available information about the indicator microorganisms for foodborne pathogens in animal wastes during thermal processing.

Bacterial Stresses and Stress-Induced Cross-Protection in *Salmonella*

Bacterial stress is generally defined as a physical, chemical, or nutritional condition insufficiently severe to kill but leading to sub-lethal injury of microbes (Wesche et al., 2009). Composting or stockpiling of animal waste may pose a significant challenge and create many hostile stresses for the survival and growth of human pathogens. Some common types of stress associated with the composting or stockpiling include desiccation, heat shock, and acid stresses (Shepherd et al., 2010; Singh et al., 2010; Singh et al., 2012).

Desiccation stress. During composting or stockpiling, moisture level in the animal waste mixture, especially at the surface of the pile, is reduced rapidly due to evaporation or the self-heating during the thermophilic phase of composting (Shepherd et al., 2007). Water loss through the desiccation process is an important factor affecting the survival and persistence of bacterial pathogens in low-water-activity environmental

habitats, such as soil, sand, and compost surface (Shepherd et al., 2010). The rdar morphotype (colonies are "red, dry, and rough" when grown on media containing Congo red), a multicellular behavior of most *S. enterica* and *E. coli* isolates, has been shown to be a major factor for non-host desiccation resistance and survival (Zaragoza et al., 2012). This morphotype is characterized by the expression of adhesive extracellular matrix components, including cellulose and curli fimbriae (Brandl et al., 2013). Both rdar and non-rdar morphotypes, including the saw (smooth and white) morphotype, can be found in nature (Solomon et al., 2005).

Heat stress. Heat shock occurs when microorganisms are exposed to sub-lethal temperatures above their normal growth limit (Farber and Brown, 1990). Temperature during composting process increases gradually, from ambient temperature to the mesophilic range and then to the thermophilic phase, which may consequently cause heat shock or stimulate a concomitant genetic and physiological heat shock response in some population of pathogenic bacteria (Shepherd et al., 2010). Especially during the extended mesophilic phase of composting, some bacterial cells may become acclimatized to sub-lethal high temperatures before lethal temperatures are reached, allowing them to survive and, in some cases, multiply under stressful conditions. In support of this notion, results of Singh et al. (2010) revealed that heat-shocked *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* at 47.5°C survived longer in dairy compost than non-heat-shocked cells at composting temperatures of 50, 55, and 60°C.

Acid stress. Acid stress can occur in low pH conditions when H^+ ions cross the bacterial cell membrane and create an acidic intracellular environment. Acid resistance is

especially crucial for foodborne pathogens that must survive the hostile acidic condition in the ruminant stomach before entering and colonizing the small intestines or colon (Berk et al., 2005). Pathogenic cells present in compost of animal origin may become acid-adapted as they are exposed to acidic condition when passing through the gastric tract.

The presence of stressed microorganisms in manure and compost could pose significant public health concerns when applying the contaminated compost to arable land. Many stressed pathogens either retain or exhibit enhanced virulence and invasion, thus making their inactivation crucial to ensure food safety (Rowe and Kirk, 2000; Humphrey, 2004). *S.* Enteritidis PT4 with enhanced heat and acid resistance has been reported to be more virulent in mice and more invasive in chickens than the non-resistant reference strain (Humphrey et al., 1996). Interestingly, some stresses that are originally part of the host's defense system are very similar to those occurred in the natural environment (Foster and Spector, 1995). Therefore, it is reasonable to speculate that pathogens may treat stresses encountered during poultry litter composting or stockpiling as a signal for the expression of virulence factors. When pathogen-contaminated poultry wastes or their composted products are used as biological soil amendments, pathogens with increased virulence could transfer to produce in the field and thus cause serious foodborne disease outbreaks. Singh and Jiang (2014) determined the gene expression of *E. coli* O157:H7 heat-shocked in dairy compost. They observed that virulence genes, such as *stx*1 and *fliC*, were up-regulated, while genes *stx*2, *eaeA*, and *hlyA* were down-regulated. And several heat shock genes were up-regulated in *E. coli* O157:H7 when

heat-shocked in dairy compost, indicating the potential induction of heat shock response during the composting process.

Cross-protection. Bacteria typically respond to stresses by altering their cellular morphology, membrane composition, biological metabolism, and virulence. Such stressed microorganisms produce a series of stress responses that can afford cross-protection against other stresses, indicating that the adaptation to a single sub-lethal stress may also enhance the tolerance to multiple lethal stresses. In fact, bacteria, especially foodborne pathogens, are frequently exposed to environmental stresses that cross-protect them against various other stresses (Wesche et al., 2009). Bacterial cells can gradually adapt to the hostile sub-lethal conditions, causing an adaptive response accompanied by a temporary physiological change that may result in an enhanced stress tolerance (Yousef and Courtney, 2003).

The general stress response identified in most Gram-negative bacteria, such as *E. coli*, *S.* Typhimurium, and *P. aeruginosa*, is regulated by the sigma factor, RpoS (σ^S) (Abee and Wouters, 1999). Induction of RpoS makes bacteria more resistant to environmental stresses, such as high and low temperatures, prolonged starvation, osmotic shock, pH stress, and oxidative stress (Suh et al., 1999). Bacteria defective in the gene (*rpoS*) for RpoS synthesis have proved to be more sensitive to different adverse conditions (Cheville et al., 1996). van Hoek et al. (2013) reported that a fully functional RpoS system can provide an advantage for the survival in the manure-amended soil environment. In their study, *E. coli* O157 isolates capable of long-term survival in manure-amended soil were all absent of mutations in *rpoS* gene; however, the strains not

capable of persisting for a long term were mutants in *rpoS* gene. RpoS integrates multiple signals (e.g. the general stress response regulators) and regulates the expressions of over 50 genes involved in the responses to various stresses. These response pathways extensively overlap and bacteria exposed to one sub-lethal stress may thus develop cross-protection against other stresses (Battesti et al., 2011). Investigating the expressions of some desiccation-associated genes may shed light on understanding the mechanisms of the cross-protection of desiccation-adapted *Salmonella* in poultry litter against subsequent heat treatment.

Summary

Poultry litter is usually recycled into the soil to improve the structure and fertility of agricultural land. As an important source of nutrients for crop production, poultry litter may possibly contain a variety of human pathogens, such as *Salmonella*, that can threaten human health upon the consumption of contaminated food or water. Thermal processing is a preferred choice for inactivating pathogens in poultry litter prior to agricultural land application. However, some populations may become acclimatized to a hostile environment during composting or stockpiling and develop heat resistance through cross-protection against subsequent high temperature treatment. The objectives of this study are to:

1) investigate the thermal inactivation of desiccation-adapted *S. enterica* in aged chicken litter,

2) study the effects of chicken litter storage time and ammonia content on thermal resistance of desiccation-adapted *S. enterica*,

3) develop a two-step heat treatment for inactivating desiccation-adapted *S. enterica* in aged chicken litter,

4) select the indigenous indicator microorganisms for validating desiccation-adapted *S. enterica* reduction in physically heat-treated poultry litter,

5) study the thermal resistance and gene expression of both desiccation-adapted and rehydrated *S.* Typhimurium in aged broiler litter.

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CHAPTER TWO

THERMAL INACTIVATION OF DESICCATION-ADAPTED *SALMONELLA* SPP. IN AGED CHICKEN LITTER

ABSTRACT

Thermal inactivation of desiccation-adapted *Salmonella* spp. in aged chicken litter was investigated in comparison with non-adapted cells to examine potential cross-tolerance of desiccation-adapted cells to heat treatment. A mixture of four *Salmonella* serovars was inoculated into the finished compost with 20, 30, 40, and 50% moisture contents for a 24-h desiccation adaptation. Afterwards, the compost with desiccation-adapted cells was inoculated into the aged chicken litter with the same moisture content for heat treatments at 70, 75, 80, 85 and 150°C. Recovery media were used to allow heat-injured cells to resuscitate. A 5-log reduction of the desiccation-adapted cells in aged chicken litter with 20% moisture content required >6, >6, 4~5, and 3~4 h exposure at 70, 75, 80, and 85ºC, respectively. As a comparison, a 5-log reduction of non-adapted cells in the same chicken litter was achieved within 1.5~2, 1~1.5, 0.5~1, and <0.5 h at 70, 75, 80, and 85ºC, respectively. Exposure time required to obtain a 5-log reduction in the desiccation-adapted cells gradually became shorter as temperature and moisture content were increased. At 150ºC, desiccation-adapted *Salmonella* survived for 50 min in chicken litter with 20% moisture content, whereas control cells were detectable by enrichment until only 10 min. Our results demonstrated that the thermal resistance of *Salmonella* in aged chicken litter was increased significantly when the cells were adapted to

desiccation. This study also validated the effectiveness of thermal processing being used for producing chicken litter free of *Salmonella* contamination.

INTRODUCTION

Chicken litter is a waste by-product of poultry production and is comprised of feces, wasted feeds, bedding materials, and feathers (37). More than 14 million tons of chicken litter is produced annually in the United States (22). Chicken litter is usually recycled as an organic fertilizer or soil amendment for direct application to agricultural land (8). However, chicken litter may contain loads of human pathogens, such as *Salmonella* spp., that have the great potential to directly or indirectly contaminate fresh produce and cause foodborne disease outbreaks (37). Currently, high temperature processing is the most commonly applied method to reduce or eliminate potential pathogens in chicken litter (15; 37).

Some microorganisms become acclimatized to desiccation stress under dry environment, and induction of desiccation stress response in bacterial cells makes them more resistant to the dry condition they are present (36). Most importantly, exposure to a single stress is found to be associated with the development of cross-tolerance to multiple unrelated stresses (10). Using laboratory models, various researchers have demonstrated that the desiccated cells exhibit increased thermal resistance (3; 10; 12). Previous thermal inactivation studies on bacterial pathogens in chicken litter have used only non-stressed cells (15; 37). Therefore, to simulate real-world conditions, thermal inactivation of desiccation-adapted cells should be evaluated as they are present in the chicken litter during stockpiling.

A population of pathogens subjected to sublethal heat treatment undergoes metabolic injury (38). Nonetheless, these sublethally injured cells present the same threat in food safety as their non-injured counterparts, because they can be resuscitated and the pathogenicity is restored under suitable conditions (20). Hence, the significance of injured pathogens should not be ignored. In a nonselective medium, the injured cells are usually repaired and then become functionally normal; however, this medium cannot differentiate target pathogen from a mixed population. On the other hand, the injured cells may fail to resuscitate when plated directly onto media with selective compounds (14). A repair step on nonselective media is therefore necessary before exposure to a selective medium for enumeration. Several studies have revealed that the presence of exogenous pyruvate in recovery media enhanced the repair of heat-stressed pathogens (1; 4; 5; 30). Busch and Donnelly (5) reported that tryptic soy broth supplemented with 1% sodium pyruvate facilitated the repair of heat-injured *Listeria monocytogenes* cells. As heat-injured *Salmonella* cells in chicken litter are potentially pathogenic, appropriate recovery methods should be incorporated into enumeration procedures. In the current study, different recovery procedures were compared and employed to allow the resuscitation of heat-injured cells and ensure that an accurate microbiological analysis could be obtained.

As far as we are aware, there have been no available reports studying the thermal inactivation of desiccation-adapted pathogens in compost and manure. We herein hypothesize that desiccation stress during storage under stockpiling conditions could possibly increase the cross-tolerance of *Salmonella* cells in chicken litter to subsequent

exposure to high temperatures. The objective of this study was thus to investigate the potential cross-tolerance of desiccation-adapted *Salmonella* spp. in the aged chicken litter to heat treatment.

MATERIALS AND METHODS

Sample preparation. Fresh chicken litter was collected from chicken barn of Bovan laying hens raised at Morgan Poultry Center, Clemson, SC, whereas the aged chicken litter was sourced from Cobb broiler chickens (Organic Farms, Livingston, CA). To prepare the aged chicken litter, the litter inside the chicken house was removed annually followed by a partial windrow composting of 7-10 d. After composting, the litter was screened out of rice hulls and ready for subsequent heat treatment. Commercially available dairy compost (Black Gold Compost Co., Oxford, FL) and poultry compost (Black Gold Compost Co., Oxford, FL) were purchased from a local supermarket. All the compost samples were dried under the fume hood until moisture contents were reduced to less than 20%, and then screened to the particle size of less than 3 mm using a sieve. Sufficient samples were collected for the entire experiment and stored in a sealed container at 4°C until use.

Bacterial strains. *Salmonella enterica* serovars Enteritidis H2292 and Heidelberg 21380 (provided by Dr. Michael Doyle, University of Georgia, Griffin, GA), Senftenberg ATCC 43845, and Typhimurium 8243 [genotype: *thyA deo polA2 zie-3024*::Tn*10*(dTc)*zag-1256*::Tn*10*(dKm), derived from *S.* Typhimurium LT2 by Dr. John Foster, University of South Alabama, Mobile, AL, and provided by Dr. Roy Curtiss III,

Washington University, St. Louis, MO)] were used for the thermal inactivation study. *S.* Typhimurium 8243 was used for the optimization of recovery media for heat-injured cells and selection of compost for desiccation adaptation. All the strains were induced to rifampin resistance (100 μg of ml^{-1}) using gradient plate method (30).

Inoculum preparation. Each *Salmonella* strain was grown overnight at 37°C in tryptic soy broth containing 100 μg of rifampin ml^{-1} (TSB-R). The overnight cultures were washed three times with sterile 0.85% saline, and the final pelleted cells were resuspended in 0.85% saline to desired cell concentrations by measuring the optical density at 600 nm.

Optimization of recovery media for heat-injured *Salmonella*. Fresh chicken litter was used for the optimization of recovery media for heat-injured *Salmonella*. The moisture content of chicken litter was adjusted to 30% (a_w, 0.932) with sterile tap water. The overnight culture of *Salmonella* was washed and resuspended in 0.85% saline to ca. 10^9 cfu ml^{-1} (an optical density of ca. 0.6~0.7 at 600 nm). Chicken litter was inoculated with *Salmonella* cells (1:100, v/w) using a sterile spray nozzle and thoroughly mixed to a final concentration of ca. 10^7 cfu g^{-1}.

About 20 g of samples were distributed evenly inside an aluminum pan (I.D. 10 cm), placed in three different locations (close to the door, center, and far away from the door) on the shelf of a controlled convectional oven (Binder Inc., Bohemia, NY), and then exposed to 75°C up to 1 h. Temperature was monitored constantly using T type thermocouples (DCC Corporation, Pennsauken, NJ), with one cord kept inside the oven chamber and others inserted into the bottom of litter samples of three different locations.

The temperature was initially set at a higher set point of 80ºC to minimize the come-up time. When the interior of the litter reached the desired temperature, the temperature setting of the oven was readjusted to maintain at the designated experimental temperature. Samples were taken out at 0.5 and 1 h, and placed immediately in an ice water bath to cool down the samples rapidly and minimize further cell death. Samples were then diluted serially with 0.85% saline and transferred in triplicate to different media to evaluate the recovery efficiency with these media. Samples taken at the beginning of heat treatment (0 h) were used to determine the initial populations. These experiments were performed in three separate trials.

Tryptic soy agar (TSA) was used as a nonselective medium, while TSA with 100 μg of rifampin ml^{-1} (TSA-R) and xylose lysine Tergitol-4 agar with 100 μg rifampin ml^{-1} (XLT-4-R) were used as selective media.

The following media were used for the recovery of heat-injured *Salmonella* cells:

1) TSA supplemented with 100 μg rifampin ml^{-1};

2) XLT-4 supplemented with 100 μg rifampin ml^{-1};

3) Modified two-step overlay (OV) method (OV/TSA-R and OV/XLT-4-R) (14): Heat-injured cells were plated directly onto TSA. After incubation at 37°C for 3 h to allow recovery of injured cells, 7 ml of TSA-R or XLT-4-R was overlaid onto TSA. Plates were incubated at 37°C for another 21 h and then colonies were counted;

4) Modified thin agar layer (TAL) method (TAL/TSA-R and TAL/XLT-4-R) (14): After solidification of 25 ml TSA-R or XLT-4-R in the plate, 14 ml of melted TSA

(48°C) was overlaid. Heat-injured cells were plated onto TAL media, which were then incubated at 37°C for 24 h;

5) TSA supplemented with 100 μg rifampin ml^{-1} and 1% sodium pyruvate (P/TSA-R);

6) TSB supplemented with 100 μg rifampin ml^{-1} and 1% sodium pyruvate (P/TSB-R): After heat treatment, 1 ml of heat-injured cells was transferred into 9 ml of P/TSB-R and incubated at 37°C for 3 h, followed by plating onto TSA-R and incubated for another 21 h.

Selection of compost matrix for desiccation adaptation of *Salmonella*. To select the compost matrix for desiccation adaptation of *Salmonella*, dairy compost, fresh poultry compost, old poultry compost, and aged chicken litter were compared based on changes in bacterial populations before and after desiccation adaptation. The overnight grown *S.* Typhimurium was washed, and resuspended in 0.85% saline to ca. 10^9 cfu ml^{-1}. To prepare inoculum for desiccation-adaptation experiments, this resuspended culture was further concentrated 100 times (ca. 10^{11} cfu ml^{-1}) by centrifuging. Afterwards, the culture was added separately (1:100, v/w) into 300 g of above four different composts with the moisture content of 30% (a_w values of dairy compost, fresh poultry compost, old poultry compost, and aged chicken litter were 0.980, 0.916, 0.938, and 0.943, respectively) at a final concentration of ca. 10^9 cfu g^{-1} for a 24-h desiccation adaptation. Before and after desiccation adaptation, samples were homogenized and serial dilutions of homogenates were plated in duplicate onto XLT-4-R for enumeration. Two trials were conducted for each experiment.

Thermal inactivation. Compost selected for desiccation adaptation and aged chicken litter samples were all adjusted to desired moisture contents, 20 (a_w, 0.873), 30 (a_w, 0.943), 40 (a_w, 0.975), and 50% (a_w, 0.986), with sterile tap water. Each of 4 *Salmonella* serotypes was grown separately overnight at 37°C, washed, and mixed in equal volume as inoculum. Both desiccation-adaptation and inoculation were performed as described above. Thermal inactivation study was carried out as described in Figure 2.1. The temperatures used for this study were 70, 75, 80, 85, and 150°C. For heat treatments at above temperatures up to 6 h, at predetermined time intervals, duplicate samples were taken out, homogenized, and plated on recovery media. The samples which were negative by direct plating recovery method (detection limit: 1.30 log cfu g^{-1}) were preenriched in universal preenrichment broth (UPB) followed by a secondary enrichment in Rappaport-Vassiliadis (RV) broth supplemented with 100 µg rifampin ml^{-1}. After 24 h incubation at 42°C, enriched samples were then plated onto XLT-4-R. Presumptive-positive colonies on XLT-4-R were further confirmed as *Salmonella* using immunolatex agglutination test (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). For heat treatment at 150°C, duplicate samples were withdrawn every 10 min up to 60 min and enriched in UPB directly to test if *Salmonella* is alive. To serve as controls, washed *Salmonella* cultures (ca. 10^9 cfu ml^{-1}) kept at room temperature for 24 h were aseptically added to aged chicken litter (20% moisture content) in a ratio of 1:100 (v/w), and exposed to above temperatures as the desiccation-adapted cultures were.

Pulsed field gel electrophoresis (PFGE). Bacterial colonies were randomly selected from the TAL recovery media with the longest survival (defined as the last

sampling time that *Salmonella* could be detected by direct-plating) in chicken litter with 20% moisture content after exposure to the heat treatment at 80ºC. The selected colonies (n=12) for each sample was transferred on TSA for two times, and then characterized by PFGE as described by CDC/PulseNet (6). Control was prepared as previously described. The band patterns of these isolates were compared with the genetic profiles of four serotypes used in this study.

Moisture content, pH, and ammonia. Moisture content was determined with a moisture analyzer (model IR-35, Denver Instrument, Denver, CO). The pH value and ammonia content in compost were measured according to the methods as described by U.S. Composting Council (33) and Weatherburn (35), respectively.

Thermal inactivation kinetics. An exponential model was used to describe the thermal inactivation of control and desiccation-adapted *Salmonella* spp. in aged chicken litter. The equation for the model is given by

$$\mathrm{Log}_{10}[N_i(t)]=\alpha+\beta e^{-\lambda t}+\varepsilon_i$$

where $N_i(t)$ is the number of bacteria at time t for the ith observation, α is the long-term ($t\rightarrow\infty$) log count of the bacteria, β is the long-term reduction in the log count of the bacteria, λ is the decay rate, and ε_i is the error for the ith observation.

It was assumed that the distribution for the number of bacteria at time t followed a log-normal distribution while taking into account the possible tailing effect. Thus, the errors were assumed to follow a normal distribution with a mean of zero and a variance of σ^2.

Statistical analysis. Plate count data were converted to log cfu g^{-1} in dry weight. SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA) was used for data analysis. Analysis of variance (ANOVA), followed by the least significant differences (LSD) test, was carried out to determine whether significant differences ($P<0.05$) existed among different treatments.

For the thermal inactivation kinetics study, the parameters for the exponential model were estimated using maximum likelihood that accounted for censored observations that were not detectable by plating. The censored observations were within the interval of 0 to 1.30 log cfu g^{-1}. Separate regression models were used for each moisture-temperature combination. Because of the censoring, a pseudo-R^2 was calculated for each regression model as described by Magee (17). The pseudo-R^2 was calculated as

$$\text{pseudo-}R^2=1-\exp[(x_2-x_1)/n]$$

where x_1=-2 log likelihood (model with no independent variables), x_2=-2 log likelihood (current model), and n is sample size.

Linear contrasts were used for all comparisons and the Type I error rate was controlled at $P=0.05$ using the Bonferroni method. The NLMIXED procedure of the Statistical Analysis System 9.3 (SAS Institute Inc., Cary, NC, USA) was used for all calculations in thermal inactivation kinetics study.

RESULTS

Fresh chicken litter, aged chicken litter, dairy compost, and poultry compost used in this study were all free of *Salmonella* by following the microbiological detection method described by US FDA's Bacteriological Analytical Manual (34).

Among eight media tested for recovering heat-injured *S.* Typhimurium, the highest populations of this pathogen was enumerated on P/TSB-R ($P<0.05$) (Table 2.1). However, the high level of microbial counts for P/TSB-R may not only be attributed to the repair of injured cells but may also reflect the multiplication of non-injured cells during the 3-h incubation. For 0.5 and 1 h heat treatments, no significant differences ($P>0.05$) in the enumeration of heat-injured *S.* Typhimurium occurred among TSA-R, XLT-4-R, OV/TSA-R, OV/XLT-4-R, TAL/TSA-R, TAL/XLT-4-R, and P/TSA-R. Among media containing TSA-R, TAL/TSA-R media recovered slightly more *Salmonella* cells, which were 3.96±0.35 and 2.80±0.39 log cfu g^{-1} at 0.5 and 1 h, respectively. With respect to media containing XLT-4-R, higher numbers of *S.* Typhimurium cells were observed on OV/XLT-4-R media, which were 3.80±0.22 and 2.75±0.45 log cfu g^{-1} at 0.5 and 1 h, respectively.

As shown in Table 2.2, *S.* Typhimurium counts in all four composts decreased during the 24-h desiccation adaptation at room temperature. The populations in fresh and old poultry composts, and aged chicken litter decreased more rapidly than those in dairy compost ($P<0.05$). The *Salmonella* reductions in dairy compost, fresh poultry compost, old poultry compost, and aged chicken litter were 0.45, 2.99, 2.18, and 2.87 log cfu g^{-1}, respectively. The levels of ammonia (average of 820.64 μg NH_4-N g^{-1}) and pH (average of 8.77) in fresh poultry compost, old poultry compost, and aged chicken litter were

much higher ($P<0.05$) compared with dairy compost (ammonia content of 22.64 μg NH_4-N g^{-1} and pH of 7.70) (Table 2.2).

The come-up times for heating aged chicken litter with different moisture contents at 70, 75, 80, 85, and 150ºC ranged from 0.42 to 2.53 h (data not shown). Our results showed that the higher initial moisture content of chicken litter required the longer come-up time. At 70, 75, 80, and 85ºC, *Salmonella* levels in aged chicken litter decreased in all samples during heat treatment; however, the difference in the populations of control and desiccation-adapted *Salmonella* was significant ($P<0.05$) (Figure 2.2). For example, at 70ºC, in aged chicken litter with the moisture content of 20%, the control cells survived for 1.5~2 h as detected by enrichment, whereas the desiccation-adapted cells survived for more than 6 h of heat exposure (Figure 2.2A). The desiccation-adapted cells were inactivated much faster when the moisture content of chicken litter was increased. For example, at 80ºC, there were still more than 2 log cfu g^{-1} counts in chicken litter with 20% moisture content after exposure to heat treatment for 6 h, whereas *Salmonella* cells survived for less than 3 h at 50% moisture content as detected by enrichment (Figure 2.2C).

At 150ºC, desiccation-adapted *Salmonella* cells still displayed extended survival as compared to the non-adapted cells (Table 2.3). Control and desiccation-adapted cells in aged chicken litter at 20% moisture content were detectable by enrichment up to 10 and 50 min, respectively. Desiccation-adapted cells in chicken litter had a shorter duration of survival with the increase of moisture content. Viable *Salmonella* cells in

chicken litter could still be detected up to 50 min at 20 and 30% moisture contents, whereas they were only detectable within 40 min at 40 and 50% moisture contents.

All the parameter estimates obtained from fitting the experimental observations into an inactivation model and the pseudo-R^2 values were shown in Table 2.4. The exponential model used in this study was appropriate for fitting all the inactivation curves and permitted the modeling of an extended tail for desiccation-adapted *Salmonella*, which was supported by the good performance in goodness-of-fit (Pseudo-R^2). α values of desiccation-adapted cells were higher as compared to those of control ($P<0.05$), which was attributed to the tailing part in the inactivation curves and thus reflected a higher level of population remaining viable at the end of thermal treatment. Meanwhile, β values of desiccation-adapted cells were lower than those of control ($P<0.05$), which suggested a lower population reduction and that they were more heat-resistant at longer exposure times. For desiccation-adapted cells, as temperature increased from 70 to 85ºC and moisture content increased from 20 to 50%, there seemed to be a trend in temperature and moisture content dependencies for these two parameters, as α values decreased while β values gradually became higher. Interestingly, the moisture content threshold to achieve a long-term log count (α value) of zero for desiccation-adapted cells decreased with an increase in temperature. At 70ºC, there was no moisture content threshold, but at higher temperatures, including 75, 80, and 85ºC, the moisture content thresholds were 50, 40, and 30%, respectively. It should be remarked that the λ values (decay rates) obtained from the inactivation model exhibited no dependencies on either non-adapted cells or

desiccation-adapted cells, temperature, or moisture content, and it is thus difficult to draw a definite conclusion from this parameter.

Colonies that exhibited the longest survival based on growth on TAL recovery media (0.5 h, and 6 and 24 h for control and desiccation-adapted cells, respectively) at 80ºC were characterized using PFGE (Table 2.5). For the non-adapted cells, 3 and 6 of 12 isolates were identified as *S.* Senftenberg and *S.* Typhimurium, respectively, whereas 7 and 3 of 12 isolates from desiccation-adapted cells were identified as *S.* Senftenberg and *S.* Typhimurium, respectively. Our results also showed that *Salmonella* could still be detected by enrichment after 24 h at 80ºC in chicken litter with 20% moisture content, with all 12 isolates identified as *S.* Senftenberg.

DISCUSSION

In order to accurately enumerate the bacterial populations after heat treatment, eight media were compared for recovering heat-injured *Salmonella* cells. Although the traditional OV method is useful for enumeration of heat-injured cells, it is difficult to isolate pure colonies on OV/XLT-4-R that grow under the selective medium. A modified TAL method (TAL/TSA-R) was also adopted to allow the isolation of colonies for further characterization with PFGE. Therefore, TAL/TSA-R and OV/XLT-4-R were selected as recovery media for following experiments on thermal inactivation of *Salmonella* spp. For data analysis, *Salmonella* populations were averaged from the counts on these two media in order to simplify the data.

Dairy compost, fresh poultry compost, old poultry compost, and aged chicken litter were compared to select the compost matrix for desiccation adaptation. Our results indicated that dairy compost was much better at maintaining *Salmonella* cells at a high population, when compared to other tested composts which contained higher ammonia contents that possibly accelerated the death of *Salmonella* in these samples. Ammonia has been reported to cause a significant reduction of human pathogens, such as *S.* Typhimurium, *Escherichia coli* O157:H7, and *L. monocytogenes*, in chicken manure (11). Therefore, dairy compost was selected as the matrix for desiccation adaptation of *Salmonella* in the following thermal inactivation experiments.

In the present study, *Salmonella* populations in aged chicken litter decreased during exposure to all tested temperatures, with shorter survival time at higher temperatures. These findings are in general agreement with other published data of the thermal inactivation of *Salmonella* spp. in chicken litter (15; 37). Wilkinson *et al.* (37) could not detect any *S.* Typhimurium in poultry litter (30, 50, and 65% moisture contents) after 1 h at 55 or 65°C in water bath. Kim *et al.* (15) found that a 7-log reduction of *Salmonella* spp. in fresh chicken litter (30, 40, and 50% moisture contents) could be achieved by heat treatment at 80°C for 44.1 to 63.0 min. The above results on temperature-time combination requirements for eliminating *Salmonella* varied among studies. This difference may be explained by differences in the composition and moisture level of compost material, *Salmonella* strains, and also heating source.

Our results clearly revealed that bacterial cells became less heat resistant when moisture content of aged chicken litter increased from 20 to 50%, which is in agreement

with the results of Kim *et al.* (15) in fresh chicken litter. Within the same duration of exposure, moist heat is known to kill microbial cells at a lower temperature than dry heat (32). Exposure to moist heat generally denatures enzymes and other essential proteins, as well as membranes and nucleic acids. Interestingly, our finding is in contrast with the results obtained by Kim *et al.* (15) who reported that *Salmonella* survived longer in aged chicken litter with 40 and 50% moisture contents than in 30%. This difference may be attributed to different chicken litter samples with various chemical compositions and physical characteristics that can result in different heat transfer kinetics. As suggested by Kim *et al.* (15), aged chicken litter used in their study had higher levels of heavy metals that might become more soluble at higher moisture content, resulting in stronger heat resistance of *Salmonella*. Additionally, our regression model analysis of thermal inactivation data for desiccation-adapted cells clearly suggests a moisture content threshold at higher temperatures. With the boosted killing rate of *Salmonella* as temperature is increased, the impact of moisture content on pathogen inactivation may reach the plateau. This information is especially critical when designing thermal processes for chicken litter with varied moisture levels.

Previously published studies on the survival of *Salmonella* exposed to heat treatment in composts have used non-stressed cultures (15; 37). Under real-world stockpiling conditions for chicken litter, *Salmonella* cells may have been exposed to the dry environment for a long period of time. It is well-known that bacteria can be induced to stress such as osmotic stress (dry environment) (36), and adaptation to a stress may also cross-protect bacterial cells against other lethal stresses. In support of this notion,

other studies have demonstrated the thermal tolerance of desiccation-adapted *Salmonella* spp. in a variety of matrix. Hiramatsu *et al.* (12) reported that desiccated *Salmonella* spp. in dried paper disk acquired the remarkable ability to survive for 5 h of exposure to 70 and 80°C. Similar results have been reported by Gruzdev *et al.* (10) who found that desiccated *Salmonella* cells in sterile deionized water showed high tolerance to dry heat at 60°C, with no significant population change within 1 h as a comparison to a 3-log reduction in non-desiccated cells under identical conditions. Also in the work of Mattick *et al.* (19), *Salmonella* cells habituated at low a_w of 0.95 for 12 h resulted in an increase in heat resistance in tryptic soy broth at 54°C, with more than a 4-fold increase in $D_{54°C}$ values.

To our knowledge, there is no information available regarding the cross-tolerance of desiccation-adapted pathogens in compost and manure to further thermal treatment. Nevertheless, heat shock response at sublethal temperature by simulating mesophilic phase of composting has been shown to provide multi-protection for *Salmonella* to composting stresses. In the field study of Shepherd *et al.* (25), heat-shocked *E. coli* O157:H7 and *S.* Typhimurium in dairy manure co-composted with vegetable wastes had extended survival in the summer composting trial. They demonstrated that the heat-shock treatment may have induced the cross-resistance of these pathogens to desiccation stress. Additionally, results of Singh *et al.* (28) suggested that heat-shocked *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* at 47.5°C survived longer than non-heat-shocked cells at composting temperatures (50, 55, and 60°C). Noticeably, Singh *et al.* (29) found acid-adaptation, a stress response being produced during exposure to animal gastric acidity,

only provided cross-protection for *E. coli* O157:H7 to thermal inactivation in saline solution; however, this protection was lost in fresh dairy compost during simulated optimal mesophilic phase of composting.

In this study, we have examined the cross-tolerance of desiccation-adapted and non-adapted *Salmonella* cells to heat treatment. Thermal inactivation curves for control and desiccation-adapted *Salmonella* cells were all non-linear regardless of temperatures and moisture contents. However, desiccation-adapted cells had significantly higher populations throughout heat treatment and also survived much longer as compared to control cultures. As compared to control, there were ca. >3, >4, 4~10, and >6 folds increases in the exposure times required for reducing 5 logs of desiccation-adapted *Salmonella* cells at 70, 75, 80, and 85°C, respectively. Presumably, one reason for this high level of thermal tolerance could be due to the fact that *Salmonella* cells were well-adapted to survive in dry condition, thereby resulting in induced cross-protection to thermal inactivation. Besides, it should be noted that pronounced tailing was observed in the survival curves of desiccation-adapted *Salmonella* at 70, 75, 80, and 85°C, which was further confirmed by our thermal inactivation kinetics study. This finding is consistent with the data published by Kim *et al.* (15), which reported that thermal inactivation curves of *Salmonella* in aged chicken litter showed extensive tailing effect. The possible explanation for the tailing is that more heat-sensitive cells were inactivated at a relatively faster rate while leaving behind survivors with higher resistance. Our observation implies that desiccation-adapted cells from the tailing in survival curves should be considered sufficiently when applying thermal treatment to chicken litter. Otherwise, inadequate

thermal processing would lead to the survival of a few heat-resistant desiccation-adapted cells, and when heat-treated chicken litter is used as organic fertilizer or soil amendment, these desiccation-adapted cells could contaminate produce in the field.

Although the mechanism of heat resistance of desiccation-adapted bacteria is still not clearly understood, the "water replacement hypothesis" could be a possible explanation. In this hypothesis, some nonreducing disaccharides such as trehalose are considered to play a pivotal role in stabilizing the structures of membranes and proteins in desiccation conditions (24). There are two genes that are supposed to be involved in the trehalose biosynthesis: *otsA* gene and *otsB* gene that encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively (7). In the study of Howells *et al.* (13), the *otsA* and *otsB* genes have been isolated from *S.* Typhimurium and nucleotide-sequenced. And they also found that after incubation at 50ºC for 7 min, the number of viable cells of a *S.* Typhimurium *otsA* mutant decreased to 0.6% of the initial population, whereas the wild type still had 8% survival rate. Further systematic experiments are warranted to explore the underlying mechanism of thermal resistance of desiccation-adapted *Salmonella* cells in chicken litter.

It is generally recommended that multiple strain composites of well-characterized pathogens should be utilized in the challenge studies on evaluating the efficacy of heat-based hurdles (27). Moreover, as also commented by others (2; 27), we hold the position that when constructing a bacterial cocktail for experiments related with food safety, strains with robust stress resistance behavior should be included. In the present study, the thermal inactivation of *Salmonella* spp. was thus investigated using four serotypes,

including three serotypes (*S.* Enteritidis H2292, *S.* Heidelberg 21380, and *S.* Typhimurium 8243) most frequently isolated from chicken litter (26) and one heat-resistant serotype (*S.* Senftenberg ATCC 43845) (18). An obvious variability in heat resistance among *Salmonella* serotypes was observed during thermal exposure of aged chicken litter, since *S.* Senftenberg and *S.* Typhimurium exhibited higher resistance profiles than the other two serotypes. To characterize the serotypes that could only be detectable by enrichment after a longer period of time, we also carried out a 24-h heat treatment at 80ºC for chicken litter with 20% moisture content. *S.* Senftenberg, the most heat-resistant *Salmonella* serotype, could even survive for up to 24 h at 80°C. At this point, a significant practical consequence is that serotypes with robust thermal inactivation characteristics, such as *S.* Senftenberg, may be used as indicator microorganisms to assure microbial risk assessment of the 'worst-case scenario' when evaluating the thermal processing of chicken litter in further heat challenge studies. In the work of Mocé-Llivina *et al.* (21), *S.* Senftenberg was selected as a suitable bacterial indicator when they assessed the thermal treatment of dewatered sludge and raw sewage. As also commented by Murphy *et al.* (23), if a particular thermal treatment destroys *S.* Senftenberg, it will also be preferably effective against more common *Salmonella* spp. In their study, *S.* Senftenberg was thus used as a test organism to determine the thermal inactivation of *Salmonella* spp. in meat products.

In summary, our results demonstrated that *Salmonella* cells adapted under desiccation condition survived substantially longer in aged chicken litter compared to non-adapted cells cells when exposed to the same heat treatment. The reduced moisture

levels in chicken litter contribute to the better survival of *Salmonella* during heat treatment. It needs to be recognized that if these surviving cells are present in chicken litter as organic fertilizer, there is a great risk for introducing cross-contamination of fresh produce before harvest. Additionally, comparison of heat resistance characteristics among 4 *Salmonella* serotypes suggested that *S.* Senftenberg can be used as an indicator microorganism for validating thermal processing of chicken litter. Our research has important implications for the chicken litter processors to validate and modify their heating process in order to eliminate *Salmonella* that may have been subjected to dry stress.

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Table 2.1 Comparison of recovery media for heat-injured *S.* Typhimurium in fresh chicken litter

Recovery media	Population of *S.* Typhimurium (log cfu g^{-1}) after exposure to 75ºC for (h)		
	0	0.5	1
TSA-R	6.71±0.06b[a]	3.83±0.38b	2.77±0.24ab
XLT-4-R	6.14±0.29c	3.74±0.45b	2.42±0.70ab
OV/TSA-R	6.69±0.15b	3.82±0.52b	2.20±0.51b
OV/XLT-4-R	6.61±0.02b	3.80±0.22b	2.75±0.45ab
TAL/TSA-R	6.68±0.12b	3.96±0.35b	2.80±0.39ab
TAL/XLT-4-R	6.19±0.42c	3.66±0.40b	2.44±0.68ab
P/TSA-R	6.69±0.18b	3.78±0.47b	2.73±0.35ab
P/TSB-R	7.59±0.33a	4.78±0.32a	3.33±0.71a

[a]Data are expressed as means±SD of three trials. Means with different letters in the same column are significantly different ($P<0.05$).

Table 2.2 Populations of *S.* Typhimurium before and after desiccation adaptation in different composts, and their pH and ammonia levels

Sample	Population of *S.* Typhimurium (log cfu g^{-1})		pH	NH_4-N (μg g^{-1})
	Before desiccation adaptation	After desiccation adaptation		
Dairy compost	8.81±0.01a[a]	8.36±0.13a	7.70±0.05d	22.64±3.22d
Fresh poultry compost	8.78±0.06a	5.79±0.14c	9.09±0.02a	1142.55±100.27a
Old poultry compost	8.76±0.04a	6.58±0.03b	8.26±0.01c	465.82±3.86c
Aged chicken litter	8.75±0.05a	5.88±0.25c	8.97±0.04b	853.55±72.64b

[a]Data are expressed as means±SD of two trials. Means with different letters in the same column are significantly different ($P<0.05$).

Table 2.3 Survival of control and desiccation-adapted *Salmonella* spp. in aged chicken litter at 150ºC

Sample	Moisture content (%)	Survival with exposure time (min)					
		10	20	30	40	50	60
Control	20	+[a]	-[b]	-	-	-	-
Desiccation-adapted cells	20	+	+	+	+	+	-
	30	+	+	+	+	+	-
	40	+	+	+	+	-	-
	50	+	+	+	+	-	-

[a]+, Detectable by enrichment.
[b]-, Not detectable by enrichment.

Table 2.4 Parameter estimates of the inactivation model for control and desiccation-adapted *Salmonella* spp.

Temperature (ºC)	Sample	Moisture content (%)	Long-term log count (α)	Long-term reduction in log count (β)	Decay rate (λ)	Pseudo-R^2
70	Control	20	-0.11±0.18B	6.98±0.32A	1.06±0.11B	0.96
	Desiccation-adapted cells	20	3.58±0.05Aa	3.35±0.12Bb	1.95±0.19Aa	0.97
		30	2.98±0.08b	3.95±0.18ab	1.67±0.18a	0.96
		40	2.50±0.21b	4.43±0.42ab	1.70±0.50a	0.83
		50	1.50±0.51b	5.00±0.51a	0.83±0.35a	0.83
75	Control	20	-0.05±0.08B	7.00±0.18A	1.49±0.09A	0.98
	Desiccation-adapted cells	20	2.39±0.09Aa	4.51±0.19Bb	1.60±0.17Aa	0.96
		30	1.62±0.35a	4.95±0.46ab	1.07±0.37a	0.84
		40	1.11±0.36a	5.43±0.46ab	1.02±0.30a	0.86
		50	0.00±0.38b	6.29±0.49a	0.75±0.17b	0.88
80	Control	20	-0.02±0.01B	7.06±0.03A	2.19±0.02A	0.99
	Desiccation-adapted cells	20	2.28±0.15Aa	4.54±0.30Bc	1.46±0.27Ba	0.92
		30	1.78±0.20a	5.14±0.39bc	1.73±0.40a	0.87
		40	0.04±0.23b	6.55±0.38ab	0.96±0.14a	0.92
		50	-0.02±0.12b	6.94±0.25a	1.26±0.11a	0.96
85	Control	20	-0.02±0.01B	7.11±0.03A	2.79±0.04A	0.99
	Desiccation-adapted cells	20	1.77±0.11Aa	5.18±0.24Bb	1.53±0.19Bab	0.95
		30	0.63±0.36b	5.98±0.50ab	1.08±0.32b	0.87
		40	-0.06±0.05b	7.12±0.11a	1.51±0.06b	0.99
		50	-0.06±0.04b	7.09±0.09a	2.06±0.07a	0.99

[a]Data are expressed as means±SE of two trials. Within each temperature, for desiccation-adapted cells, means with different lowercase letters in the same column are significantly different (P<0.05), while means of control and desiccation-adapted cells (20% moisture content) with different uppercase letters in the same column are significantly different (P<0.05).

Table 2.5 Characterization by PFGE of *Salmonella* spp. (n=12) in aged chicken litter with 20% moisture content after thermal inactivation at 80ºC

Sample	Serotype
Control (after 0.5-h heat treatment)	*S.* Enteritidis (n=2[b]), *S.* Heidelberg (n=1), *S.* Senftenberg (n=3), *S.* Typhimurium (n=6)
Desiccation-adapted cells (after 6-h heat treatment)	*S.* Enteritidis (n=2), *S.* Heidelberg (n=0), *S.* Senftenberg (n=7), *S.* Typhimurium (n=3)
Desiccation-adapted cells (after 24-h heat treatment)	*S.* Enteritidis (n=0), *S.* Heidelberg (n=0), *S.* Senftenberg (n=12), *S.* Typhimurium (n=0)

[a]Number of colonies.

Figure 2.1 Flow chart of the experimental procedure.

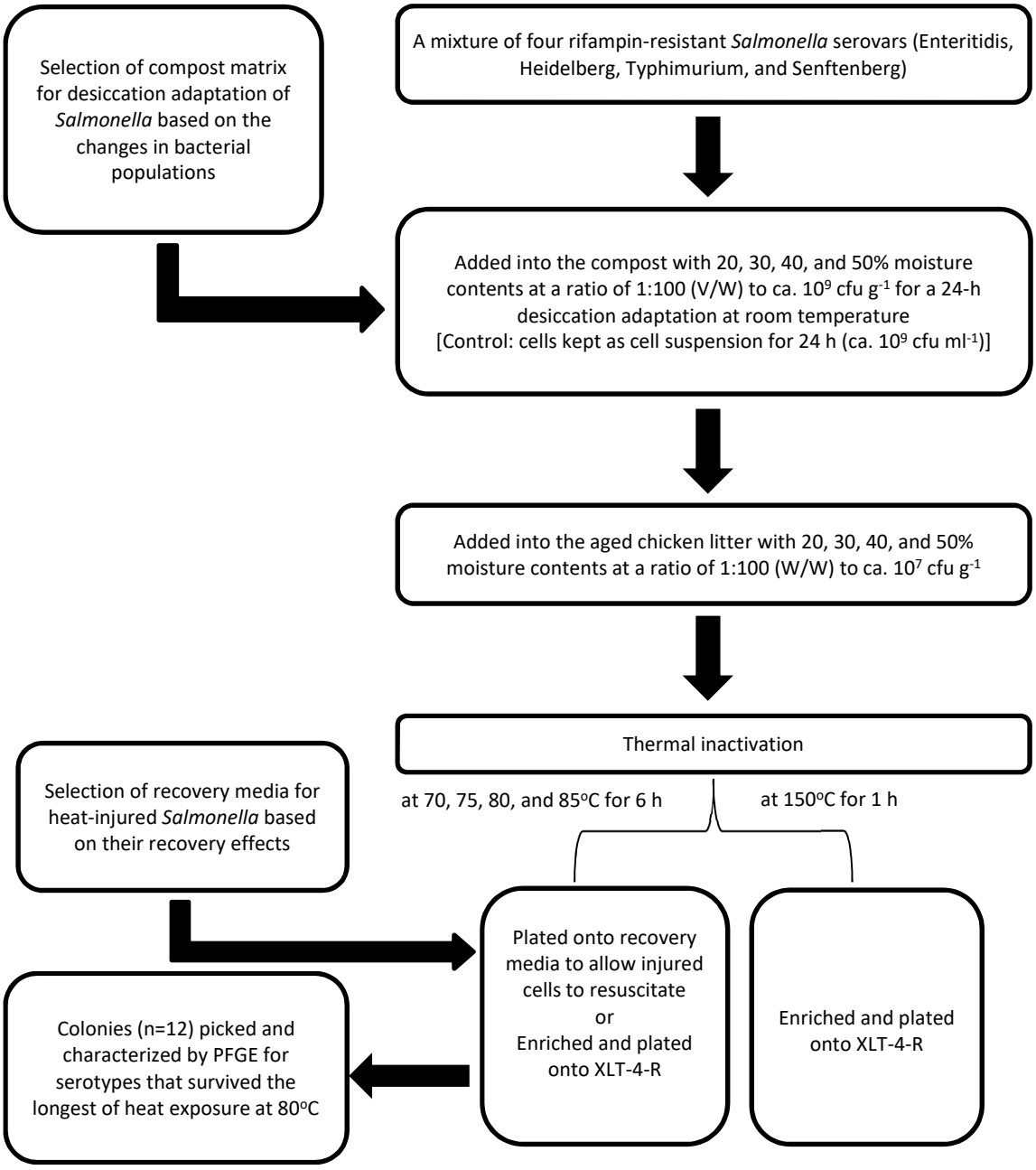
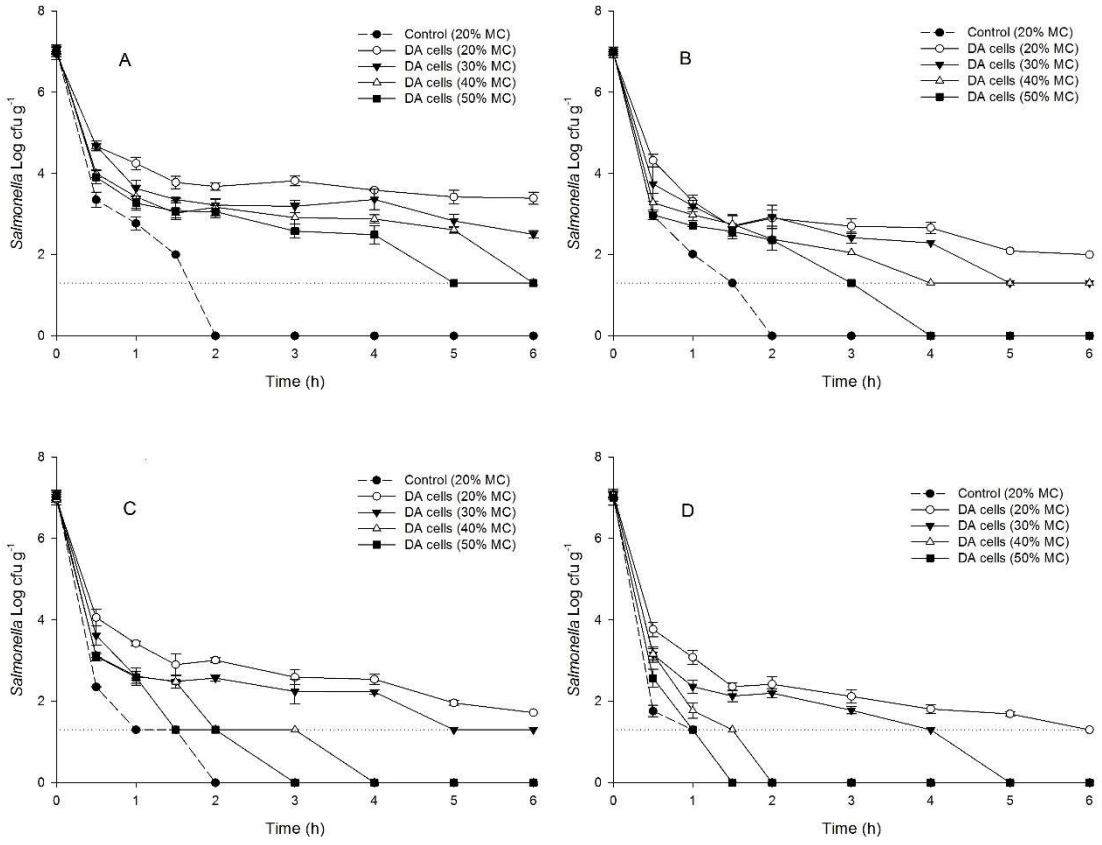


Figure 2.2 Survival of control and desiccation-adapted (DA) *Salmonella* spp. in aged chicken litter with 20, 30, 40, and 50% moisture contents (MCs) at (A) 70, (B) 75, (C) 80, and (D) 85ºC. The dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by plating: 1.30 log cfu g^{-1}).



CHAPTER THREE

EFFECTS OF CHICKEN LITTER STORAGE TIME AND AMMONIA CONTENT ON THERMAL RESISTANCE OF DESICCATION-ADAPTED *SALMONELLA* SPP.

ABSTRACT

Broiler chicken litter was kept as a stacked heap on a poultry farm and samples were collected up to 9 months of storage. Chicken litter inoculated with desiccation-adapted *Salmonella* cells was heat-treated at 75, 80, 85, and 150°C. *Salmonella* populations decreased in all these samples during heat treatment, and the inactivation rates became slower in chicken litter when storage time was extended from 0 to 6 months. There was no significant difference (P>0.05) in thermal resistance of *Salmonella* in 6- and 9-month litter samples indicating that a threshold for thermal resistance was reached after 6 months. Overall, the thermal resistance of *Salmonella* in chicken litter was affected by the storage time of the litter. The changes in some chemical, physical, and microbiological properties during storage could possibly contribute to this difference. Moisture and ammonia could be two of the most significant factors influencing the thermal resistance of *Salmonella* cells in chicken litter. Our results emphasize the importance of adjusting time-temperature conditions for heat processing chicken litter when it is removed from the chicken house at different time intervals.

INTRODUCTION

Chicken litter is a waste by-product of poultry industry, which consists of feces, bedding materials, wasted feeds, and feathers (1). More than 14 million tons of chicken

litter are produced every year in the U.S., most of which are spread onto arable land as organic fertilizer or soil amendment (2). Chicken litter is known to potentially harbor a variety of human pathogens, such as *Salmonella* (3). Therefore, the direct application of chicken litter to agricultural land can possibly be harmful to the environment and also food supply (4).

Chicken litter is introduced into arable land either immediately after its removal from chicken house, being stockpiled for an extended period of time, or after going through a composting process (5). Agricultural practices on handling chicken litter vary widely among farms and also among economic crops grown on an individual farm. Chicken litter cleanout does not always coincide with available agricultural land or with suitable field conditions that allow operation of equipment or desirable nutrient uptake. Temporary storage of chicken litter should thus be provided until conditions are proper for direct application on land or chicken litter can be composted or further heat processed. Notably, chicken litter is a heterogeneous waste product with variable compositions and differences in physical, chemical, and microbiological properties, which can be affected by storage time and some other factors (6). In addition to composting, physical dry-heat treatment after composting or without composting is one of the most commonly used methods to eliminate potential pathogens in chicken litter (6). The storage time of chicken litter may, to some extent, affect the thermal inactivation of foodborne pathogens. Based on the study of Kim et al. (6), *Salmonella* survived much longer in dry or aged chicken litter as compared to wet or fresh litter when exposed to the same lethal temperature, suggesting that the moisture level and chemical composition

(especially ammonia) of chicken litter are critical factors affecting the thermal resistance of this pathogen. However, in their study, the origins of fresh and aged chicken litter samples were different, which could place some limitations on the application of their findings.

It is known that chicken litter is a relatively hostile environment for the growth and persistence of enteric pathogens, since it is generally very dry and high in ammonia (5). However, some pathogenic cells may become acclimatized to hostile environments during stockpiling or composting, which can cross-protect them against subsequent thermal treatment (1; 7). A limitation regarding most published thermal inactivation studies on pathogens in compost and manure is that the conclusions are primarily based on using non-stressed cells (5; 6), and the data obtained from these studies can be misinformed or biased. Currently, the use of stressed cells in the challenge studies on validating the efficacy of heat-based hurdles for foodborne pathogens is still novel, and few examples of quantitative assessments are available in the published literature (8; 9). Our previous work has already demonstrated that desiccation-adapted *Salmonella* cells in chicken litter were more heat resistant than non-adapted populations during dry-heat treatment, suggesting possible survival of some heat-resistant desiccation-adapted cells due to inadequate thermal processing (8; 9). These pathogenic cells can potentially contaminate fresh produce and environment, when physically heat-treated chicken litter is applied to agricultural land as organic fertilizer or soil amendment. This concern has created a significant, industry-wide demand for establishing some practical guidelines on temperature-time combination to thermally process chicken litter of different properties

to produce the finished products as *Salmonella*-free. Therefore, desiccation-adapted cells should be used in order to simulate the real-world conditions in chicken litter during composting or stockpiling.

The objective of this study was to determine the thermal resistance of desiccation-adapted *Salmonella* spp. as affected by the changes in some physical, chemical, and microbiological characteristics of chicken litter during long-term storage. We believe that this novel study will elucidate the effects of various properties of stacked chicken litter on die-off dynamics of desiccation-adapted *Salmonella* spp., and provide some important new insights into the microbiological safety of heat-processed chicken litter as biological soil amendment for agricultural application.

MATERIALS AND METHODS

Sample preparation. The chicken litter was removed from the broiler house at Mendel Stone Farm, Westminster, SC after harvesting all the chickens. The litter was kept as a stacked round heap (ca. 1.2 m in height × 2 m in diameter) under a covered shed on the farm for 9 months (May, 2013~February, 2014). The shed was a typical roofed storage structure commonly seen on poultry farms, as described by Ogejo and Collins (10). The internal (ca. 50 cm from the surface) and external temperatures of the heap ranged from -2.5 to 32°C and from -15 to 34°C, respectively.

Chicken litter samples were collected aseptically at 0, 3, 6, and 9 months of storage. Subsamples of 10 kg (n=5) were taken at different locations (internal and

external) throughout the heap. These subsamples were thoroughly mixed using a clean shovel and placed into sample bags. Sufficient samples (ca. 30 kg) were collected for the entire experiment, screened manually to the particle size of less than 3 mm using a sieve (sieve pore size, 3 × 3 mm), and then stored at 4°C until use.

Bacterial strains and inoculum preparation. *Salmonella enterica* serovars Enteritidis H2292 and Heidelberg 21380 (kindly provided by Dr. Michael Doyle, University of Georgia, Griffin, GA), Senftenberg ATCC 43845, and avirulent Typhimurium 8243 [genotype: *thyA deo polA2 zie-3024*::Tn*10*(dTc)*zag-1256*::Tn*10*(dKm), derived from *S.* Typhimurium LT2 by Dr. John Foster, University of South Alabama, Mobile, AL, and provided by Dr. Roy Curtiss III, Washington University, St. Louis, MO)] (8) were used. All the strains were induced to rifampin resistance (100 μg of ml^{-1}) using gradient plate method (11).

Each *Salmonella* strain was grown overnight at 37°C in tryptic soy broth without dextrose (TSB; Becton, Dickinson and Company, Sparks, MD) supplemented with 100 μg of rifampin/ml. The overnight cultures were centrifuged and washed three times with sterile phosphate buffered saline (PBS; pH, 7.4). The final pelleted cells were resuspended in PBS to desired cell concentrations (ca. 10^9 cfu/ml) through adjusting the optical density at 600 nm to ca. 0.7. Afterwards, these resuspended cultures were further concentrated 100 times (ca. 10^{11} cfu/ml) by centrifugation. Equal volumes of four cultures were mixed as inoculum for the subsequent experiments.

Desiccation adaptation and thermal inactivation. Briefly, as described by Chen et al. (8), bacterial cultures were added (1:10, v/w) into 100 g of chicken litter collected at

different storage times at a final concentration of ca. 10^{10} cfu/g, mixed well, and incubated at room temperature for a 24-h desiccation adaptation. After the 24-h desiccation adaptation, chicken litter with desiccation-adapted cells was further mixed (1:10, w/w) with 800 g of chicken litter using a blender (KitchenAid Inc., St. Joseph, MI) for subsequent heat treatment.

Twenty grams of samples in triplicate were distributed evenly inside an aluminum pan (I.D. 10 cm), placed in three different locations (close to the door, center, and far away from the door) on the shelf of a controlled convectional oven (Binder Inc., Bohemia, NY), and then exposed to 75, 80, and 85°C for up to 6 h. Temperature was monitored constantly using T type thermocouples (DCC Corp., Pennsauken, NJ), with one cord kept inside the oven chamber and others inserted into the bottom of litter samples of three different locations. The temperature of the oven was initially set 5°C higher than the target temperature to reduce the come-up time. When the interior of litter sample reached target temperatures, temperature setting was readjusted to maintain at the designated experimental temperature. Chicken litter samples were taken out of the oven every 0.5 h, transferred into a Whirl-Pak bag (Nasco, Fort Atkinson, WI), and placed immediately in an ice-water bath to cool down the samples and minimize further cell death. For heat treatment at 150°C, triplicate samples were withdrawn every 15 min for up to 1 h. These experiments were performed in two separate trials.

***Salmonella* enumeration.** For the challenge study, ten grams of samples were mixed with 90 ml of sterile PBS (pH, 7.4) containing 1% sodium pyruvate, kept at room temperature for 30 min to allow recovery of heat-injured cells (12), and then diluted

serially with PBS. Afterwards, diluents were spread in triplicate onto TSA supplemented with 100 µg rifampin/ml. In case of the possible failure of recovering any heat-injured cells by direct plating method (detection limit: 1.52 log cfu/g) after 1 h of heat treatment, 25 g of chicken litter samples was pre-enriched in 225 ml of universal pre-enrichment broth (UPB; Neogen Corp., Lansing, MI) followed by a secondary enrichment in Rappaport-Vassiliadis broth (RV; Becton, Dickinson and company, Sparks, MD) supplemented with 100 µg rifampin/ml. After 24-h incubation at 42°C, enriched cultures were then streaked with a 10 µl inoculation loop onto xylose lysine tergitol agar (XLT-4; Becton, Dickinson and company, Sparks, MD) supplemented with 100 µg rifampin/ml. Samples taken at the beginning of heat treatment (0 h) were used to determine the initial inoculum levels. For heat treatment at 150°C, samples were plated onto TSA supplemented with 100 µg rifampin/ml and also enriched as described above to test if there were viable *Salmonella* cells.

Thermal inactivation kinetics. A log-linear model with shoulder and/or tailing was used to describe the thermal inactivation of desiccation-adapted *Salmonella* spp. in chicken litter (13), which can be formulated as follows

$$\frac{\mathrm{d}N}{\mathrm{d}t} = -k_{\max} \cdot N \cdot \left(\frac{1}{1 + C_{\mathrm{C}}}\right) \cdot \left(1 - \frac{N_{\mathrm{res}}}{N}\right)$$

$$\frac{\mathrm{d}C_C}{\mathrm{d}t} = -k_{\max} \cdot C_{\mathrm{C}}$$

N is the microbial cell density [log cfu/g], t is the treatment time [h], C_c is related to the physiological state of the cells, k_{max} is the specific inactivation rate [1/h], and N_{res} is the residual population density [log cfu/g].

The model was fitted using averages from two experimental data sets. An adjusted regression coefficient R^2 was used to evaluate the goodness-of-fit of the proposed model as described by Chen and Zhu (14).

Analyzing physical, chemical, and microbiological characteristics of chicken litter. Moisture content was determined using a moisture analyzer (model IR-35, Denver Instrument, Denver, CO), whereas water activity (a_w) was measured with a dew-point water activity meter (Aqualab series 3TE, Decagon Devices, Pullman, WA). Ammonia content and pH value were measured according to the methods as described by Weatherburn (15) and U.S. Composting Council (16), respectively. Electrical conductivity was determined using the Orion™ VERSA STAR™ conductivity meter (Thermo Fisher Scientific Inc., Waltham, MA) based on the method described by U.S. Composting Council (16). Each sample was analyzed in duplicate. Duplicate samples were analyzed for chemical characteristics, including nutrients and metals (total concentrations, including water-soluble and water-insoluble concentrations), based on the methods proposed by U.S. Composting Council (16).

Prior to the challenge study, chicken litter was analyzed for background microorganisms, including mesophiles, thermophiles, *Enterobacteriaceae*, yeasts and molds, and actinomycetes, and also screened for *Salmonella* by following the microbiological detection method described by U.S. FDA's Bacteriological Analytical

Manual (17). The media used for mesophiles, thermophiles, *Enterobacteriaceae*, yeasts and molds, and actinomycetes were tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD), TSA, Violet red bile dextrose agar (VRBD; Becton, Dickinson and Company, Sparks, MD), Rose-Bengal chloramphenicol agar (Oxoid Ltd., Basingstoke, UK), and Actinomycete isolation agar (Becton, Dickinson and Company, Sparks, MD), respectively, while the incubation temperatures used for them were 35, 55, 35, 22, and 30ºC, respectively, and the incubation times were 24, 24, 24, 72, and 72 h, respectively.

The metabolic fingerprint pattern for microbial community in chicken litter was investigated with the EcoPlate™ method (Biolog Inc., Hayward, CA). The EcoPlate™ was designed specifically for microbial community analysis and microbial ecological studies. The EcoPlate™ contains 31 of the most useful carbon sources for community analysis. Communities of microorganisms will display a characteristic reaction pattern called a metabolic fingerprint, which rapidly and readily provides a vast amount of information from the MicroPlate. Litter samples were diluted in sterile PBS (pH, 7.4) to reach a bacterial concentration of background microflora of approximately 10^3 cfu/ml, and then wells with different carbon sources and 3 blanks were inoculated. Plates were placed inside a plastic container with lid containing moist towels and incubated at room temperature for up to 7 days. The development of purple color was monitored by measuring optical density at 590 nm every 24 h using the μQuant microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT). The data collection was

performed on day 5, since there was no further significant color formation after that time point.

Statistical analysis. Plate count data were converted to log cfu/g in dry weight. Differences among samples were determined by least significant differences (LSD) using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA) and were considered to be significant when $P<0.05$. For the study on the microbial community in chicken litter, data analysis was performed with Principal Component Analysis (PCA) using XLSTAT 2010 (Addinsoft Inc., New York, NY) to compare and analyze the changes in the microbial community reaction patterns.

RESULTS AND DISCUSSION

Effect of storage time of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. Before artificial inoculation, chicken litter samples were all free of *Salmonella* by following the microbiological detection method described by US FDA's Bacteriological Analytical Manual (17). To determine the effect of chicken litter storage time on thermal resistance of *Salmonella*, all chicken litter samples were inoculated with desiccation-adapted *Salmonella* cells at the level of ca. 7.5 log cfu/g. At 75, 80, and 85°C, *Salmonella* populations decreased in all these samples during heat treatment; however, the inactivation rates became slower in chicken litter when storage time was extended from 0 to 6 months (Figure 3.1). For example, at 75°C, *Salmonella* cells were only detectable by enrichment after 2 h of heat exposure for 0-month litter,

whereas up to 4 logs of *Salmonella* were recovered following 6-h heat treatment for 6- and 9-month litter samples (Figure 3.1A). Interestingly, there was no significant difference in the thermal resistance of *Salmonella* in chicken litter with storage times of 6 and 9 months ($P>0.05$), indicating that a threshold for thermal resistance was reached after 6 months. In the chicken litter samples with the same storage time, desiccation-adapted cells were inactivated much faster when temperature was increased. For example, for 9-month litter, the population of *Salmonella* cells was still greater than 3.5 log cfu/g after 6 h of heat exposure at 75°C; however, viable cells could only be detected by enrichment after 5.5 h at 85°C. In addition, as compared to fresh chicken litter at day 0, the increased thermal resistance of *Salmonella* in aged chicken litter was more pronounced at lower temperatures of 75 and 80ºC than at 85ºC ($P<0.05$).

At 150ºC, *Salmonella* cells in 3-, 6-, and 9-month litter had longer durations of survival as compared to those in 0-month litter during 1-h heat treatment (Table 3.1). After 15 min of heat exposure, the populations of *Salmonella* in 6- and 9-month litter samples were significantly higher than those in 0- and 3-month litter samples ($P<0.05$). Viable cells were not detectable by enrichment after 45 min of heat treatment in 0-month litter, whereas for 3, 6, and 9-month litter samples, they could still be detected by enrichment for at least 1 h.

Parameter estimates obtained from fitting the experimental observations into the log-linear model with shoulder and/or tailing and the adjusted R^2 values are presented in Table 3.2. The model used in this study was proved to be adequate in fitting all the inactivation curves and allowed modeling of an extended tail for desiccation-adapted

Salmonella, which was supported by the good performance of goodness-of-fit experiments (Adjusted R^2). Our results showed that there seemed to be a trend in temperature and storage time dependencies for k_{max} (specific inactivation rate). As temperature decreased from 85 to 75°C and storage time extended from 0 to 9 months, k_{max} tended to decrease, suggesting a slower population reduction, i.e., a lower inactivation rate. Moreover, there also seemed to be a trend in storage time dependency for N_{res} (residual population density), as N_{res} gradually increased when storage time extended from 0 to 9 months, which reflected a higher level of population remaining viable (tail) at the end of thermal treatment. For the same storage times of 0 and 3 months, N_{res} remained relatively the same when temperature was increased from 75 to 85°C, suggesting less impact of these temperatures on those heat-resistant populations. However, when the storage time was extended from 6 to 9 months, 2 logs more of heat-resistant populations were inactivated at 85°C as compared to 75 and 80°C.

Effects of moisture of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. During the extended on-farm storage, chicken litter in stockpile was subject to changes in physical, chemical, and microbiological characteristics. In this study, moisture content of chicken litter decreased gradually from 29.93 to 18.20% after 9-month storage (Table 3.3). One of the factors contributing to the increased thermal resistance of *Salmonella* from 0 to 9 months could be the low moisture content in aged chicken litter, as bacteria can generally survive much longer when being heat-treated under dry condition (8). The results of our recent research also indicated that desiccation-adapted *Salmonella* cells in aged chicken litter became more heat-resistant

when moisture content decreased from 50 to 20% (8; 9). In the study of Kim et al. (6), *Salmonella* survived much longer in chicken litter with lower moisture content during dry-heat treatment. Ceustermans et al. (18) also observed a higher survival rate of *S.* Senftenberg when the moisture content of the composting materials or meat carriers was reduced. And similarly, Turner (19) found that higher moisture content of wheat straw and pig manure also played a positive role in enhancing the inactivation of *E. coli* during composting. When 10 ml of *E. coli* broth was added, inactivation occurred more rapidly at both 50 and 55°C as compared to when only 1 ml was added.

To exclude the possibility that decreased moisture content solely contributed to the increased thermal resistance of *Salmonella* with extended storage time, our research expanded on the above studies by comparing the survival profiles of *Salmonella* in 0- and 9-month chicken litter via adjusting the moisture content of 9-month litter (Adjusted MC; a_w, 0.90±0.00) to the same level as that of 0-month litter at 85 and 150°C (Figure 3.1C and Table 3.1). At 85°C, the population of *Salmonella* in 9-month litter with adjusted moisture content was higher than that in 0-month litter for at least the initial 2 h of heat treatment (P<0.05) while lower than that in 9-month litter without the moisture content adjustment for at least the initial 2.5 h of heat treatment (P<0.05) (Figure 3.1C). Similarly, at 150°C, *Salmonella* in 9-month litter with adjusted moisture content survived for at least the initial 15 min longer as compared to those in 0-month litter but shorter than those in 9-month litter without the moisture content adjustment throughout thermal exposure (P<0.05) (Table 3.1). Therefore, it should be noted that in addition to moisture

content, there could be some other explanations for the difference in the thermal resistance of *Salmonella* in chicken litter during heat exposure among four storage times.

Effects of ammonia of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. Ammonia level was much higher in fresh chicken litter than in aged chicken litter ($P<0.05$), as the initial high ammonia level (279.13 μg NH_4-N/g) in 0-month litter decreased to 56.37 μg NH_4-N/g in 9-month litter (Table 3.4). Generally, chicken litter, high in nitrogen content, begins to decompose immediately after excretion and releases ammonia in high concentrations (20). Chicken litter also gives off ammonia due to the production of ammonia from microbial mineralization of organic nitrogen compounds in the litter at the beginning of stockpiling (21). Ammonia can cause a significant inactivation of non-spore-forming pathogens in a stacked manure heap (22; 23). In the current study, ammonia content of chicken litter decreased rapidly throughout 9-month storage. Therefore, the initial high ammonia level could result in a large amount of ammonia emission during heat treatment, accelerating the inactivation of *Salmonella* in 0-month litter. Similar results have been obtained by Kim et al. (6) who reported that *Salmonella* cells in aged chicken litter with lower level of ammonia showed longer survival as compared to those in fresh chicken litter. Himathongkham and Riemann (24) evaluated the effect of drying and/or exposure to ammonia on *S.* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* in chicken manure. Drying to 10% moisture content followed by being exposed to ammonia gas for 72 h (equivalent to 1% of the manure wet weight) led to an 8-log reduction of *S.* Typhimurium and *E. coli* O157:H7, and 4-log reduction for *L. monocytogenes*. Thus, the dramatically decreased ammonia level in

chicken litter with extended storage time could be another possible explanation for the increased thermal resistance of *Salmonella* cells.

Effects of indigenous microbial community of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. Chicken litter was also subject to a gradual decline in mesophiles, thermophiles, and *Actinomycetes*, while *Enterobacteriace* was not detectable after 3 months (Table 3.3). The microbial community changes in chicken litter were also analyzed for metabolic profiles using an EcoPlate^{TM} method. Only six carbon sources (Tween 40, glycogen, N-acetyl-D-glucosamine, D-glucosaminic acid, glucose-1-phosphate, and D, L-α-glycerol phosphate) oxidized at a higher rate by microbial communities in chicken litter during 9-month storage (Changes in OD_{590} for 0-, 3-, 6-, and 9-month samples were all higher than 0.4) were used for PCA analysis. As shown in Table 3.5, these six carbon sources were all oxidized at a relatively higher rate. Obviously, Tween 40, glycogen, N-acetyl-D-glucosamine, D-glucosaminic acid, and glucose-1-phosphate were oxidized at the highest rate by 9-month microflora (P<0.05), while D, L-α-glycerol phosphate was oxidized at the highest rate by 3-month microflora (P<0.05). Consistently, the cluster diagram based on the EcoPlate^{TM} data also revealed a distinct clustering of the oxidization rates of these above six carbon sources in relation to storage times (Figure 3.2). Therefore, our results showed that there were some changes in the metabolic fingerprint patterns of microbial communities during 9-month storage of chicken litter.

The use of carbon sources present in the EcoPlate^{TM} was sensitive enough to detect changes in the microbial diversity in response to different storage times. By using

the PCA analysis, the different use of carbon sources allowed separation of for 0-, 3-, 6-, and 9-month samples. However, it should not be emphasized on particular sources, since carbon sources present in the EcoPlate™ do not accurately reflect the sources present in the real-world chicken litter ecosystems; so they cannot be interpreted as "*in situ*" utilization. Therefore, the carbon sources in the EcoPlate™ only provide a series of compounds that permit estimation of relative potential metabolic versatility and rapid microbial community fingerprinting.

The aged chicken litter with reduced microbiological diversity and species richness might be more susceptible to the invasion by *Salmonella* due to the lack of competition (24). Millner et al. (25) reported that the inactivation of *Salmonella* during composting was not because of the activity of a single group of microorganisms, indicating that the concentrations and diversity of background microorganisms in compost may affect the fate of this pathogen. In this study, the microbial community analysis of chicken litter revealed the changes in microbial species and population during 9-month storage; however, it is difficult to draw a conclusion about the impact on thermal resistance of *Salmonella* in chicken litter as the chicken litter was exposed to external heat source rather than the heat generated by indigenous microbial activities.

Effects of heavy metals of chicken litter on thermal resistance of desiccation-adapted *Salmonella* spp. In addition to macro- and micro-nutrients, chicken litter also contains different levels of heavy metals which are related to their additions in the diet or environmental source (26). Table 3.4 presents data on the concentrations of five common heavy metals, such as Zn, Cu, Mn, Fe, and Al. In the present study, the total

concentrations of these five heavy metals in chicken litter increased and reached the maximum at 6 months, while decreased thereafter. When Sims and Wolf (27) analyzed results from studies conducted from 1969 to 1992, they found that Zn and Cu concentrations in poultry litter ranged from non-detectable to 669 μg/g and non-detectable to 1,003 μg/g, respectively. In this study, Cu concentrations determined throughout the 9-month storage were all within the range reported by Sims and Wolf (27), although Zn concentrations became slightly higher after 3 months. Hsu and Lo (28) studied the influence of composting on the concentrations of Cu, Mn, and Zn in swine manure. Total metal concentrations increased with time, while the water-soluble fractions of metals increased at the beginning of the 122-day composting and then gradually decreased at the end. Paré et al. (29) also found that the concentrations of Zn, Cr, Cu, and Pb increased by 145, 124, 73.6, and 26.3% during 41-day co-composting of biosolids and municipal solid wastes, respectively, whereas the concentration of Co decreased by 60% and that of Ni maintained relatively constant. The difference between results of other studies and ours may be due to the fact that the duration of the stockpiling in our study was much longer and also the difference in the composition of compost material. In this study, the heavy metal concentrations reached the highest level at 6-month sampling time. Accordingly, desiccation-adapted *Salmonella* in chicken litter became increasingly resistant to lethal temperatures when storage time prolonged from 0 to 6 months. However, there was no significant difference between heat resistance characteristics of *Salmonella* in 6- and 9-month chicken litter ($P>0.05$) even though the 9-month chicken litter had lower moisture content and ammonia than those in the 6-

month chicken litter. Therefore, our findings at least implied that heavy metals could possibly be one of the factors enhancing the thermal resistance of *Salmonella* cells in chicken litter. Further study is needed to delineate the role of heavy metals in the thermal resistance of microorganisms in manure and compost.

We acknowledge the limitation in this study for using two replicates per trial in our experimental design. Therefore, the results should be interpreted with some caution, especially when taking the heterogeneity of chicken litter into consideration.

In conclusion, our results revealed that the thermal resistance of desiccation-adapted *Salmonella* in chicken litter was affected by storage times. Moisture and ammonia could be two of the most significant factors influencing the thermal resistance of *Salmonella* cells in chicken litter. This is a novel study in which we have demonstrated that the desiccation-adapted *Salmonella* cells in fresh chicken litter are more susceptible to thermal inactivation as compared to those in aged chicken litter, highlighting the importance of considering the storage time of chicken litter as a critical factor when chicken litter is heat-processed. Our recommendation to chicken litter processing industry is to thermally process chicken litter immediately after removal from chicken houses to decrease the development of heat resistance in *Salmonella* spp.

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Table 3.1 Survival of *Salmonella* spp. in chicken litter stored for 0, 3, 6, 9, and 9 (Adjusted MC) months at 150ºC

Storage time (Month)	Survival (log cfu/g) with exposure time (min)			
	15	30	45	60
0	C1.50±0.91b[a]	+[b]	-[c]	-
3	2.16±0.21b	+	+	+
6	3.29±0.28a	+	+	+
9	A3.63±0.28a	+	+	+
9 (Adjusted MC[d])	B 2.36±0.34	+	+	-

[a]Data are expressed as means±SD of two trials. Means with different lowercase letters are significantly different (P<0.05), while for 0, 9, and 9 (Adjusted MC) months, means with different uppercase letters are significantly different (P<0.05).
[b]+, detectable by enrichment.
[c]-, not detectable by enrichment.
[d]Adjusted MC, moisture content of 9-month litter was adjusted to the same level as that of 0-month litter.

Table 3.2 Parameter estimates of the thermal inactivation model for desiccation-adapted *Salmonella* spp. in chicken litter

Temperature (ºC)	Storage time (Month)	k_{max}[a]	N_{res}	Adjusted R^2
75	0	0.17	1.59	0.9709
	3	0.11	1.72	0.9080
	6	0.12	3.95	0.9131
	9	0.08	3.95	0.8843
80	0	0.18	1.57	0.9720
	3	0.13	1.61	0.9715
	6	0.13	3.88	0.9030
	9	0.12	3.92	0.8974
85	0	0.31	1.58	0.9868
	3	0.17	1.66	0.8688
	6	0.11	1.88	0.9293
	9	0.07	1.52	0.9244

[a]k_{max} is the specific inactivation rate [1/h] and N_{res} is the residual population density [log cfu/g]. Adjusted R^2 was used to evaluate the goodness-of-fit of the model.

Table 3.3 Moisture content, electrical conductivity, and microbial counts of chicken litter during 9-month storage

Storage time (Month)	Moisture content (%)	a_w	Electrical conductivity (ms/cm)	Mesophiles (log cfu/g)	Thermophiles (log cfu/g)	Enterobacteriaceae (log cfu/g)	Yeasts and molds (log cfu/g)	Actinomycetes (log cfu/g)
0	29.93±0.00a[a]	0.88±0.00a	20.25±0.36a	7.80±0.07a	7.66±0.19a	3.10±0.21	3.86±0.12a	5.03±0.32a
3	19.81±0.09b	0.79±0.00b	19.12±0.48b	6.78±0.33b	6.77±0.33b	-[b]	3.12±0.01c	3.59±0.12c
6	19.53±0.08b	0.74±0.01c	17.18±0.36c	6.66±0.07b	4.84±0.01c	-	3.43±0.15b	3.94±0.12b
9	18.20±0.00c	0.74±0.00c	15.95±0.09d	6.55±0.12b	4.10±0.23d	-	3.68±0.15a	3.40±0.20c

[a]Means with different letters in the same column are significantly different (P<0.05).
[b]-, not detectable (detection limit of 1.52 log cfu/g).

Table 3.4 Chemical characteristics of chicken litter during 9-month storage

Storage time (Month)	Nutrient (%)										Heavy metal (μg/g)					pH	NH_4-N (μg/g)
	OM[b]	C	N	C/N	P	K	Ca	Mg	S	Na	Zn	Cu	Mn	Fe	Al		
0	72.35± 0.07a[a]	36.68± 0.21a	3.72± 0.01a	9.85± 0.01b	4.10± 0.11c	3.98± 0.11c	2.89± 0.05c	0.79± 0.03c	0.88± 0.02b	0.86± 0.02c	605.00± 14.14c	180.50± 4.95c	670.50± 16.26c	2768.50 ±48.79c	3832.50± 6.36c	8.74 ±0.02a	279.13 ±11.92a
3	67.65± 0.50b	33.98± 0.57b	3.32± 0.01c	10.24± 0.23a	4.78± 0.06b	4.74± 0.15b	3.26± 0.08b	0.94± 0.04b	1.03± 0.03a	1.00± 0.01a	744.00± 18.39a	301.00± 32.53a	829.00± 28.28a	3734.00 ±288.50 b	5274.00± 309.71b	8.61 ±0.01b	98.01 ±0.26b
6	63.65± 2.48c	32.70± 0.29c	3.52± 0.06b	9.30± 0.08c	5.21± 0.35a	4.89± 0.08a	3.58± 0.11a	1.01± 0.03a	1.01± 0.13a	1.03± 0.01a	761.00± 25.46a	305.50± 6.36a	881.50± 44.55a	4068.00 ±429.92 a	6197.50± 286.38a	8.56 ±0.00c	65.05 ±0.35c
9	68.30± 1.13b	33.70± 0.54b	3.83± 0.10a	8.80± 0.38d	4.56± 0.25b	4.45± 0.13b	3.13± 0.16b	0.89± 0.04b	0.84± 0.04b	0.93± 0.02b	672.00± 8.49b	225.50± 4.95b	754.50± 34.65b	3428.50 ±577.71 b	4884.50± 740.34b	8.31 ±0.01d	56.37 ±0.28d

[a]Data are expressed as means±SD of two samples. Means with different letters in the same column are significantly different (P<0.05). The values of nutrients and metals are all calculated based on dry-weight.
[b]OM, organic matter.

Table 3.5 Changes in OD_{590} for six carbon sources after 5 days based on EcoPlate™ data for chicken litter stored for 0, 3, 6, and 9 months

Storage time (Month)	C2[b]	C5	C12	C13	C14	C15
0	0.601±0.064b[a]	0.478±0.011d	0.929±0.040d	0.532±0.021c	0.674±0.052c	0.682±0.127b
3	0.766±0.105b	1.550±0.009b	1.789±0.040b	0.920±0.078b	0.935±0.055b	0.923±0.093a
6	0.599±0.076c	1.165±0.045c	1.503±0.061c	0.578±0.082c	0.616±0.005d	0.650±0.103b
9	1.063±0.014a	1.994±0.045a	2.138±0.004a	1.428±0.021a	0.996±0.020a	0.623±0.009b

[a]Data are expressed as means±SD of three samples. Means with different letters in the same column are significantly different ($P<0.05$).

[b]C2, Tween 40, C5, glycogen, C12, N-acetyl-D-glucosamine, C13, D-glucosaminic acid, C14, glucose-1-phosphate, and C15, D, L-α-glycerol phosphate.

Figure 3.1 Survival of *Salmonella* spp. in chicken litter stored for 0, 3, 6, and 9 months at 75 (A), 80 (B), and 85ºC (C). The dash line in (C) shows the survival of *Salmonella* spp. in chicken litter stored for 9 months (Adjusted MC, moisture content of 9-month litter was adjusted to the same level as that of 0-month litter) at 85ºC. The dotted line indicates that *Salmonella* was only detectable by enrichment (detection limit by plating: 1.52 log cfu/g).

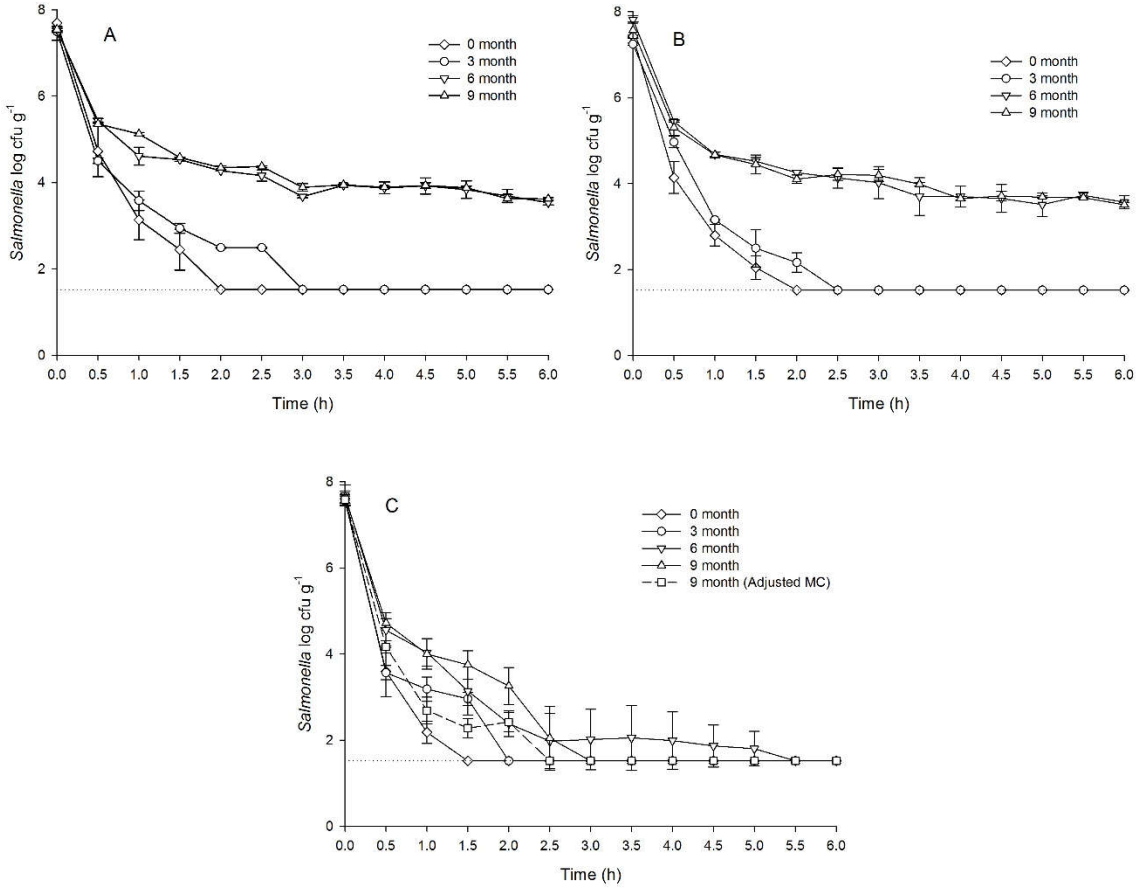
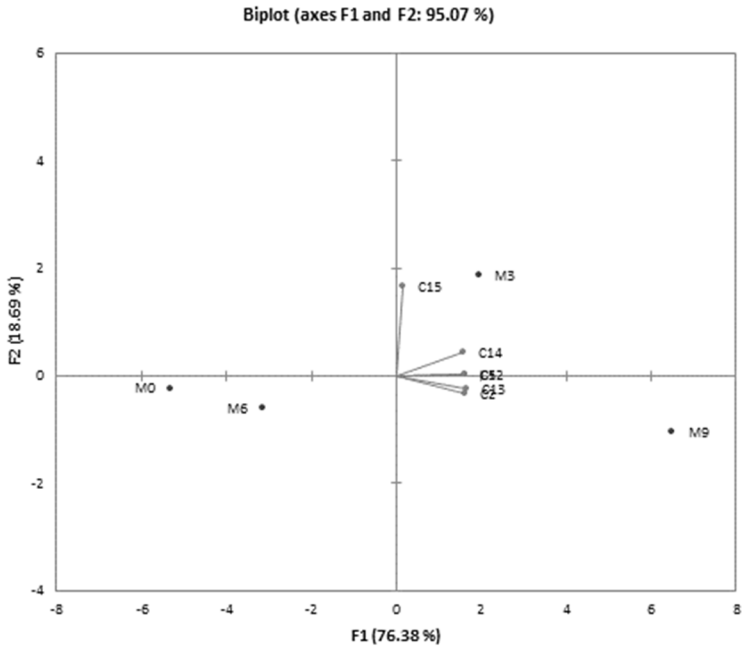


Figure 3.2 Principal component analysis (PCA) ordination diagram of EcoPlate™ data for six carbon sources (C2, Tween 40, C5, Glycogen, C12, N-Acetyl-D-Glucosamine, C13, D-Glucosaminic Acid, C14, Glucose-1-Phosphate, and C15, D, L-α-Glycerol Phosphate) oxidized at the highest rates (Changes in OD_{590}>0.7 after 5 days based on EcoPlate™ data) by microbial communities in chicken litter stored for 0 (M0), 3 (M3), 6 (M6), and 9 (M9) months.



CHAPTER FOUR

DEVELOPING A TWO-STEP HEAT TREATMENT FOR INACTIVATING DESICCATION-ADAPTED *SALMONELLA* SPP. IN AGED CHICKEN LITTER

ABSTRACT

The effectiveness of a two-step heat treatment for eliminating desiccation-adapted *Salmonella* spp. in aged chicken litter was evaluated. The aged chicken litter with 20, 30, 40, and 50% moisture contents was inoculated with a mixture of 4 *Salmonella* serotypes for a 24-h adaptation. Afterwards, the inoculated chicken litter was added into the chicken litter with the adjusted moisture content for a 1-h moist-heat treatment at 65°C and 100% relative humidity inside a water bath, followed by a dry-heat treatment in a convectional oven at 85°C for 1 h to the desired moisture level (<10~12%). After moist-heat treatment, the populations of *Salmonella* in aged chicken litter at 20 and 30% moisture contents declined from ca. 6.70 log cfu/g to 3.31 and 3.00 log cfu/g, respectively. And after subsequent 1-h dry-heat treatment, the populations further decreased to 2.97 and 2.57 log cfu/g, respectively. *Salmonella* cells in chicken litter with 40 and 50% moisture contents were only detectable by enrichment after 40 and 20 min of moist-heat treatment, respectively. Moisture contents in all samples were reduced to <10% after 1-h dry-heat process. Our results demonstrated that the two-step heat treatment was effective in reducing >5.5 logs of desiccation-adapted *Salmonella* in aged chicken litter with moisture content at or above 40%. Clearly, the findings from this study may provide chicken litter processing industry with an effective heat treatment method for producing *Salmonella*-free chicken litter.

INTRODUCTION

Chicken litter, a by-product of poultry industry, is a mixture of feces, bedding materials, waste feeds, and feathers removed from poultry houses (Bolan *et al.*, 2010). The litter has a high nutritional value for supporting plant growth, containing essential nutrients such as nitrogen (N), phosphorus (P), and potassium (K), and also trace elements, such as copper (Cu) and zinc (Zn) (Kelleher *et al.*, 2002). Most of the litter produced by poultry industry is currently applied to agricultural land, which is a feasible way to recycle the nutrients when managed correctly. However, chicken litter is also a source of human pathogens, such as *Salmonella*, that may potentially contaminate fresh produce and environment after land application, and may lead to foodborne outbreaks (Wilkinson *et al.*, 2011).

Physical dry-heat treatment after composting or without composting is one of the most commonly used methods to destroy potential pathogens in chicken litter and to produce a stable organic fertilizer (Kim *et al.*, 2012). However, some pathogenic cells may become acclimatized to sublethal conditions during mesophilic composting or stockpiling, cross-protecting them against subsequent exposure to lethal temperatures (Singh *et al.*, 2010; Chen and Jiang, 2014). Our previous study has demonstrated that desiccation-adapted *Salmonella* in aged chicken litter showed extended survival during dry-heat treatment (Chen *et al.*, 2013). Desiccation-adapted *Salmonella* cells in aged chicken litter with the moisture content of 50% could still be detected by enrichment after 40 min of dry-heat treatment even at 150°C. As a result, dry heat takes a long time to inactivate heat-resistant cells in chicken litter, suggesting that current thermal processing

techniques may not rapidly eliminate pathogens from physically heat-treated chicken litter. And these surviving pathogenic cells could potentially contaminate produce and environment, when physically heat-treated chicken litter is applied to agricultural land as organic fertilizer or soil amendment. Furthermore, prolonged thermal exposure may utilize more energy and also negatively affect the quality of chicken litter, since mechanical drying can potentially cause nutrient loss, such as nitrogen (N) loss from ammonia volatilization (Moore *et al.*, 1995).

To achieve a rapid destruction of pathogens in physically heat-treated chicken litter while minimizing quality loss, additional approaches should be explored as another hurdle for pathogen control. It is generally recognized that moist heat is a more efficient lethal treatment for microorganisms as compared to dry heat (Willey, 2008). When moist air is used to inactivate bacterial cells, much lower temperatures are required for bacterial inactivation, compared with heating with dry air. Moist heat kills microorganisms by employing water molecules to degrade nucleic acids, denature enzymes and other proteins, and disrupt cell membranes, as compared with dehydration and oxidation effects of dry heat. In animal feed industry, steam is used to condition the feed mash for rapid pathogen inactivation prior to pelletizing process (Jones, 2011). However, the response of various pathogens in animal wastes to moist heat has not been thoroughly studied.

In order to completely eliminate pathogens in chicken litter, it would be plausible to design a two-step heat treatment with the first step using moist heat to rapidly inactivate large populations of pathogens in chicken litter, and then apply dry heat to eliminate the remaining cells and to reduce the moisture content to the desired level

(<10~12%). Therefore, the objective of this study was to evaluate a two-step heat treatment for effectively eliminating *Salmonella* in aged chicken litter.

MATERIALS AND METHODS

Aged chicken litter preparation. To prepare aged chicken litter, the litter inside the Cobb broiler chicken house (Organic Farms, Livingston, CA) was collected annually followed by a partial windrow composting for 7-10 d. After composting, the litter was screened out of rice hulls. In the lab, chicken litter samples were air-dried overnight under the fume hood to reduce the moisture content to <20%, screened to the particle size of less than 3 mm using a sieve, and then stored in sealed containers at 4°C until use.

Bacterial strains. *Salmonella enterica* serovars Enteritidis H2292 and Heidelberg 21380 (kindly provided by Dr. Michael Doyle, University of Georgia, Griffin, GA), Senftenberg ATCC 43845, and Typhimurium 8243 (derived from *S.* Typhimurium LT2 by Dr. Russell Maurer, Case Western Reserve University, Cleveland, OH, and kindly provided by Dr. Roy Curtiss III, Washington University, St. Louis, MO) (Chen *et al.*, 2013) were used for the two-step heat treatment. All the strains were induced to rifampin resistance (100 μg ml^{-1}) using the gradient plate method (Smith *et al.*, 1982).

Inoculum preparation. Each *Salmonella* strain was grown overnight at 37°C in 1 L of tryptic soy broth (TSB, Dickinson and company, Sparks, MD) supplemented with 100 μg rifampin ml^{-1}. The overnight cultures were centrifuged and washed three times with sterile 0.85% saline. The final pelleted cells were resuspended in saline to desired cell concentrations (ca. 10^9 cfu ml^{-1}) through adjusting the optical density at 600 nm to

ca. 0.7. Afterwards, these resuspended cultures were further concentrated 100 times (ca. 10^{11} cfu ml^{-1}) by centrifuging. Equal volumes of four cultures were mixed as inoculum for the subsequent two-step heat treatment.

Preparation of desiccation-adapted *Salmonella* cells. .Aged chicken litter used for desiccation adaptation with the initial ammonia content of 853.55±72.64 μg g^{-1} was exposed to greenhouse conditions for 15 d to lower the ammonia content to 78.32±6.21 μg g^{-1} in order to minimize the population reduction during desiccation adaptation. The chicken litter with lower ammonia content was adjusted to the desired moisture contents of 20, 30, 40, and 50% with sterile tap water. *Salmonella* cultures were added (1:100, v/w) into 300 g of aged chicken litter with lower ammonia content at a final concentration of ca. 10^9 cfu g^{-1} for a 24-h adaptation at room temperature. Afterwards, the aged chicken litter inoculated with desiccation-adapted cells was mixed (1:100, w/w) in a mixer (KitchenAid Professional HD, KitchenAid Inc., St. Joseph, MI) with the aged chicken litter with the adjusted moisture content. Controls were washed *Salmonella* cells (ca. 10^9 cfu ml^{-1}), suspended in saline, and kept at room temperature (22ºC) for 24 h that were then added to the aged chicken litter with 20% moisture content in a ratio of 1:100 (v/w).

Two-step heat treatment. About 20 g of inoculated aged chicken litter were distributed evenly in an aluminum pan (I.D. 10 cm) in a thin layer (ca. 0.5 cm in depth), placed into a metal tray (13×9×2 inches) immersed in a water bath with water temperature set at 70ºC (Precision Scientific Inc., Chicago, IL), and treated by moist heat for 1 h. The temperature inside the litter samples reached 65ºC during the moist-heat

treatment. The relative humidity (RH) in the water bath chamber was constantly monitored with a USB data logger (EL-USB-2-LCD, Lascar Electronics Inc., Erie, PA). Then, litter samples were immediately dry-heated in a convectional oven (Binder Inc., Bohemia, NY) set at 85°C for 1 h to the desired moisture content of <12%. Temperature was initially set at a higher set point of 100ºC to minimize the come-up time for dry-heat treatment. Temperature was determined with T type thermocouples (DCC Corporation, Pennsauken, NJ), with one cord inserted into litter samples throughout two-step treatment and another cord exposed to the air inside the water bath or the oven. During dry-heat treatment, duplicate samples were taken out at 30 and 60 min, and placed immediately in an ice water bath. Samples were then homogenized in sterile 0.85% saline with a ratio of 1:10 (v/w) using a Stomacher 400 Circulator (Seward Ltd., West Sussex, UK) at medium speed (230 rpm) for 1 min. Homogenates were then diluted serially with saline.

Enumeration of *Salmonella* cells. The surviving *Salmonella* cells were enumerated using a modified two-step overlay method with Xylose-Lysine-Tergitol 4 agar (XLT-4, Dickinson and company, Sparks, MD) supplemented with 100 µg rifampin ml^{-1} as the selective media to allow heat-injured cells to resuscitate (Chen *et al.*, 2013). Litter samples collected at the beginning (0 h) were used to determine the initial counts of *Salmonella*. Samples which were negative for *Salmonella* by direct plating recovery method (detection limit: 1.30 log cfu g^{-1}) were pre-enriched in universal pre-enrichment broth (UPB, Neogen Corp., Lansing, MI) followed by a secondary enrichment in Rappaport-Vassiliadis broth (RV, Dickinson and company, Sparks, MD) supplemented

with 100 μg rifampin ml^{-1}. After 24-h incubation at 42°C, enriched cultures were then plated onto XLT-4 supplemented with 100 μg rifampin ml^{-1}.

Moisture content, a_w, ammonia, and microbiological analysis. Moisture content was determined using a moisture analyzer (Model IR-35, Denver Instrument, Denver, CO), whereas water activity (a_w) was measured with a dew-point water activity meter (Aqualab series 3TE, Decagon Devices, Pullman, WA). Ammonia content was measured according to the method as described by Weatherburn (1967). The aged chicken litter used in this study was free of *Salmonella* by following the procedures for microbiological analyses recommended by U.S. FDA's Bacteriological Analytical Manual (USFDA, 2014). Each sample in duplicate was analyzed.

Statistical analysis. Each experiment was performed in two separate trials. Plate count data were converted to log cfu g^{-1} in dry weight. Differences among samples were determined by least significant differences (LSD) using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA), and were considered to be significant when $P<0.05$.

RESULTS

The a_w of aged chicken litter increased as moisture content increased from 20 to 50% (Table 4.1). During two-step heat treatment, the temperature in aged chicken litter increased more rapidly in samples with lower moisture contents (Figure 4.1). During moist-heat treatment of chicken litter with 20, 30, 40, and 50% moisture contents, RH in the water bath increased from 52 to 100% within 15 min. During moist-heat treatment, the come-up times for heating aged chicken litter with 20, 30, 40, and 50% moisture

contents to reach the target temperature of 65°C were 13, 15, 18, and 37 min. During dry-heat treatment, the come-up times for heating aged chicken litter with 20, 30, 40, and 50% moisture contents to reach the target temperature of 85°C were 30, 45, 60, and 78 min, respectively. Obviously, the higher initial moisture content of chicken litter required the longer come-up time. Moisture levels in all samples decreased slightly during moist-heat treatment, but after 1-h drying process, dropped dramatically from the initial moisture contents of 20, 30, 40, and 50% to 4.1, 4.6, 6.2, and 8.3%, respectively, which were lower than the desired level of 12% (Figure 4.2).

Desiccation-adapted and non-adapted *Salmonella* cells in aged chicken litter were subject to two-step heat treatment. Due to the impact of heat exposure during the extended come-up times on microbial inactivation, the thermal inactivation data were collected during this period of time as well. *Salmonella* counts in aged chicken litter decreased in all samples during two-step heat treatment; however, desiccation-adapted *Salmonella* displayed extended survival as compared to the non-adapted cells in chicken litter with 20% moisture content (Table 4.1). Control cells in aged chicken litter were not detectable by enrichment after 20 min of moist-heat treatment. In contrast, desiccation-adapted *Salmonella* cells exhibited a much longer duration of survival and the population of viable desiccation-adapted cells was 2.97 log cfu g^{-1} after two-step heat treatment.

Although *Salmonella* counts in aged chicken litter decreased in all treatments during two-step heat treatment, the reductions in desiccation-adapted *Salmonella* populations in aged chicken litter with different moisture contents varied (Table 4.1). Based on our results, the desiccation-adapted cells were more quickly inactivated in aged

chicken litter samples at higher moisture contents. For aged chicken litter with 40 and 50% moisture contents, moist heat treatment for 1 h was sufficient to achieve a >5.5-log reductions of *Salmonella*.

To investigate the possibility that desiccation adaptation of *Salmonella* in aged chicken litter with low moisture content could result in increased thermal resistance, our research expanded on the above studies by comparing the survival profiles of *Salmonella* cells adapted in aged chicken litter at 20% moisture content with at 40 and 50% moisture contents during two-step heat treatment in aged chicken litter with the moisture contents of 40 and 50% (Table 4.1). Our results showed that *Salmonella* cells desiccation-adapted in aged chicken litter at 20% moisture content were inactivated much slower as compared to adaptation at 40 and 50% moisture contents.

DISCUSSION

The raw or partially composted chicken litter is currently processed by dry heat in the commercial settings. Although various organizations and federal agencies provide some guidelines to ensure effective heat treatment for animal manure, there are still no defined heating sources (dry vs. moist heat) or scientifically validated temperature-time requirements (Chen and Jiang, 2014). It is generally recognized that moist heat is a more efficient lethal treatment for microorganisms as compared to dry heat. For example, Wilkinson *et al.* (2011) reported that *S.* Typhimurium in fresh chicken litter containing rice hulls with 30-65% moisture levels was completely eliminated within 1 h at both 55

and 65ºC in a water bath. In another study, a greater than 5-log reduction of *S.* Typhimurium population in chicken litter containing pine shavings was achieved when exposed to steam for 30 or 100 min (Cox *et al.*, 1986). Therefore, in this study, we evaluated a two-step heat treatment, consisting of a moist-heat treatment for 1 h at 65°C and a sequential dry-heat treatment for 1 h at 85°C, for rapidly eliminating *Salmonella* in aged chicken litter.

As far as we are aware of, there are no published reports studying the effect of moist-heat treatment on desiccation-adapted pathogens in compost or animal manure. Therefore, in the present study, survival profiles of desiccation-adapted and non-adapted *Salmonella* cells during two-step heat treatment were compared. Our results showed that desiccation-adapted cells survived much longer in comparison to the non-adapted cells ($P<0.05$) (Table 4.1). In order to provide temperature-time recommendations for processing physically heat-treated chicken litter, the heat-resistant form of *Salmonella*, i.e. desiccation-adapted cells, was used to simulate the worst-case scenario. This high level of thermal tolerance of desiccation-adapted *Salmonella* cells could be attributed to the fact that some bacterial cells were sufficiently adapted to the hostile dry condition, which induced cross-protection to subsequent thermal inactivation by moist- and dry-heat treatments (Potts, 1994).

Our results clearly demonstrated the impact of moisture content of chicken litter on the thermal inactivation of *Salmonella* (Table 4.1). It has been postulated that water molecules that are in close contact with proteins inside a cell could be a factor influencing the microbial inactivation (Doesburg *et al.*, 1970). As expected, surviving

populations of *Salmonella* observed in the present study became lower with the increase in moisture content (consistent with the increase in a_w in this study) of chicken litter during moist-heat treatment, indicating that increased moisture content enhanced the lethal effect. Similar results have been reported earlier by Riemann (1968) who found that a drastic reduction in viable *Salmonella* population could be obtained by heating meat and bone meal at 90°C for a relatively short time after the meal was conditioned from the "natural" a_w of 0.6-0.7 to an a_w of about 0.9. And in the work of Archer *et al.* (1998), for any temperature of 57-70°C, the heat resistance of inoculated *S.* Weltevreden increased, as the initial a_w of flour prior to heating decreased from 0.6 to 0.2. Therefore, the amount of available water in chicken litter can considerably influence the effectiveness of thermal processing and, in addition to temperature and time, a_w or moisture content in chicken litter prior to heating should be considered as another critical controlling factor during two-step heat treatment. It should be noted that regarding moist heat, it is critical for the moisture to penetrate among particles of chicken litter. Therefore, it is of great significance for fertilizer processors to reduce the heterogeneity of chicken litter to ensure that a thorough processing can be achieved to eliminate all pathogenic cells.

Pelletization with heating and dehydration process involved has been widely used in poultry waste processing (Cox *et al.*, 1986; López-Mosquera *et al.*, 2008). Pelletization may increase the bulk density and the uniformity of particle size in chicken litter, allowing a higher level of nutrient density for land application (McMullen *et al.*, 2005). In pelletizing industry, regardless of heating source, temperature, and equipment, pellets

should leave the die at temperature of 60-95°C and moisture content of 12-18% (Kaddour and Alavi, 2010). In general, for long-term storage, the final moisture content of the pellets should be less than 12-13% (Robinson, 1984; Maier *et al.*, 1992). For biosolids, U.S. Environmental Protection Agency (USEPA) suggests that biosolids should be dried by direct or indirect contact with hot gas to reduce the moisture content to 10% or lower (USEPA, 2003). In this study, there was no dramatic decrease in the *Salmonella* population during dry-heat treatment. Therefore, the dry-heat treatment is mainly utilized to reduce the moisture content of chicken litter to below 12%, since moisture contents of all litter samples were reduced to <10 % after drying process, which guarantees the stability of physically heat-treated chicken litter products for long-term storage.

In conclusion, our results demonstrated that the two-step heat treatment was effective in reducing heat-resistant desiccation-adapted *Salmonella* in aged chicken litter. The higher initial moisture contents in chicken litter contributed to rapid killing of *Salmonella* during moist-heat treatment. Based on our results, a two-step heating process consisting of a moist-heat treatment for 1 h at 65°C and a sequential dry-heat treatment for 1 h at 85°C can be sufficient for achieving >5.5-log reductions of *Salmonella* in chicken litter with moisture content of ≥40%. In order to be used by chicken litter processing industry, further pilot study of this two-step heat processing is needed. And the effect of moist-heat treatment on the product quality of chicken litter should also be investigated.

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Table 4.1 Survival of desiccation-adapted *Salmonella* spp. in aged chicken litter during two-step heat treatment

Moisture content of aged chicken litter for desiccation adaptation (%)	Moisture content of aged chicken litter for heat treatment (%)	a_w	*Salmonella* population (log cfu g^{-1}) after heat treatment for (min)					
			Moist-heat treatment (1st step)				Dry-heat treatment (2nd step)	
			0	20	40	60	30	60
			Control					
20	20	0.87	6.71±0.22a[a]	-	-	-	-	-
			Desiccation-adapted *Salmonella*					
20	20	0.87	6.68±0.18a	3.75±0.12a	3.42±0.22a	3.31±0.15a	3.04±0.13a	2.97±0.27a
	40	0.98	6.71±0.21a	1.63±0.22c	+	-	-	-
	50	0.99	6.69±0.17a	1.51±0.16c	-	-	-	-
30	30	0.94	6.74±0.23a	3.25±0.08b	3.02±0.31a	3.00±0.13a	2.64±0.06b	2.57±0.16a
40	40	0.98	6.72±0.20a	+[b]	+	-[c]	-	-
50	50	0.99	6.67±0.14a	+	-	-	-	-

[a]Data are expressed as means±SD of two trials. Means with different letters in the same column are significantly different ($P<0.05$).

[b]+, detectable by enrichment.

[c]-, not detectable by enrichment.

Figure 4.1 Change of temperature in aged chicken litter during two-step heat treatment. MC, moisture content.

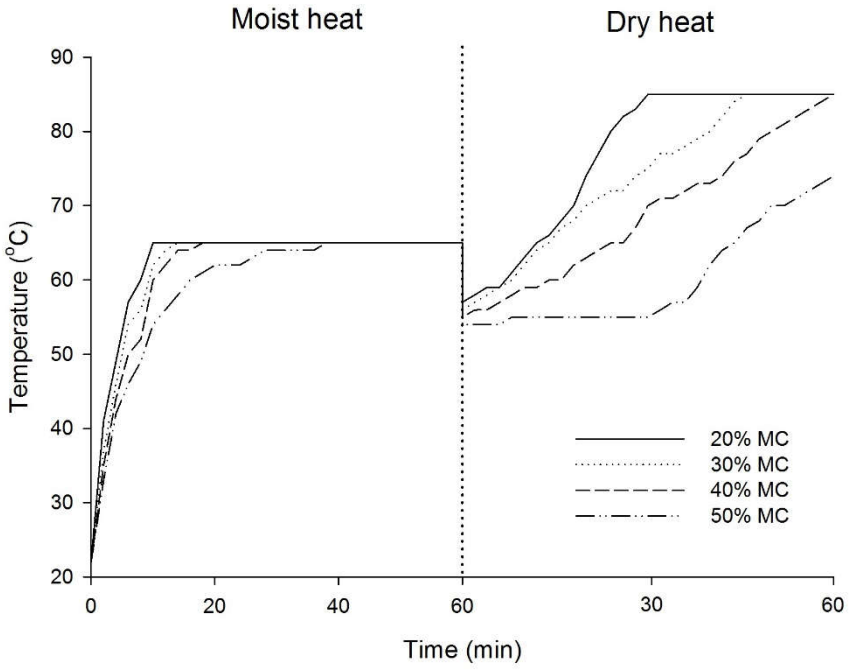
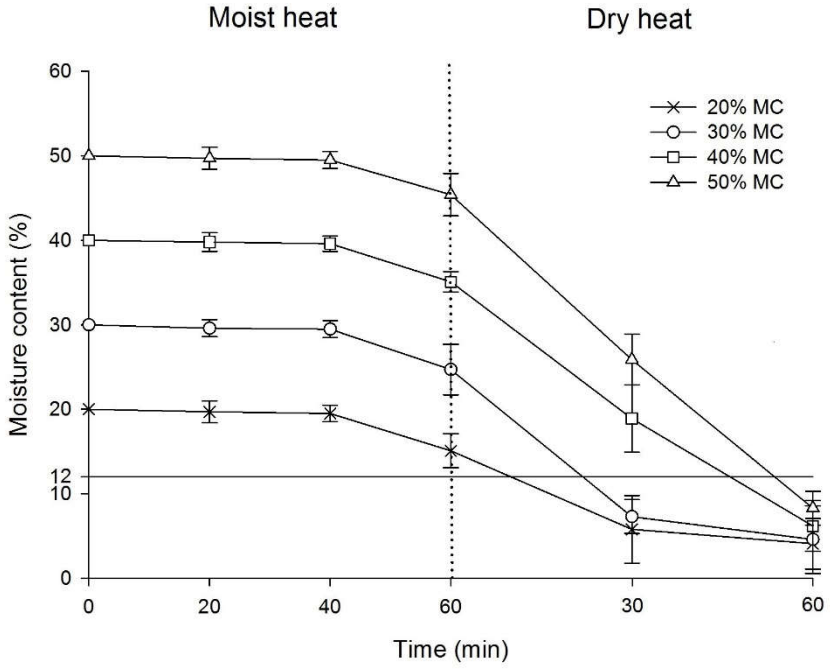


Figure 4.2 Change of moisture content in aged chicken litter during two-step heat treatment. MC, moisture content. The horizontal solid line represents the target moisture content (<12%) to reach after two-step heat treatment.



CHAPTER FIVE

SELECTION OF INDIGENOUS INDICATOR MICROORGANISMS FOR VALIDATING DESICCATION-ADAPTED *SALMONELLA* REDUCTION IN PHYSICALLY HEAT-TREATED POULTRY LITTER

ABSTRACT

The indigenous indicator microorganisms for validating desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter was selected. The thermal resistance of desiccation-adapted *Salmonella* Senftenberg 775/W was compared with those of indigenous enterococci and total aerobic bacteria in poultry litter. Aged broiler litter and composted turkey litter with 20, 30, 40, and 50% moisture contents were inoculated with desiccation-adapted *S.* Senftenberg 775/W, and then heat-treated at 75 and 85ºC. Compared to total aerobic bacteria, there were better correlations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci in broiler litter samples with 20, 30, 40, and 50% moisture contents at 75ºC (R^2>0.91), and 20, 30, and 40% moisture contents at 85ºC (R^2>0.87). The mean log reductions of *S.* Senftenberg 775/W were better-correlated with those of indigenous enterococci in turkey litter samples with 20, 30, 40, and 50% moisture contents at 75ºC (R^2>0.88), and 20 and 30% moisture contents at 85ºC (R^2=0.83) than those of total aerobic bacteria, which had a better correlation in turkey litter sample with 40% (R^2=0.98) moisture content at 85ºC. Indigenous enterococci may be used to validate the thermal processing of poultry litter as it mimics the survival behavior of *Salmonella* under some treatment conditions. This study provides some scientific data for poultry litter processors when validating the effectiveness of thermal processing.

INTRODUCTION

According to the U.S. Department of Agriculture (USDA) National Agricultural Statistics Service's 2015 Census of Agriculture, the U.S. produced more than 8.5 billion broilers and 2.3 billion turkeys in 2015 (USDA 2015). Since broilers and turkeys produce about 1.7 and 18 pounds of litter per bird, respectively (West Virginia Regional Planning and Development Councils 2012), it is estimated that the broilers and turkeys in the U.S. would have produced approximately 6.5 and 18.8 million tons of litter in 2015, respectively. Poultry litter, rich in macro- and micro-nutrients, has a great value as soil amendment and organic fertilizer for agricultural production. Nonetheless, poultry litter is also the source of some human pathogens, such as *Salmonella enterica* (Chen and Jiang 2014).

Poultry litter can be used in organic farming under certain conditions; however, under the Organic Materials Review Institute (OMRI)/USDA National Organic Program (NOP) rules and California Leafy Green Marketing Agreement, the use of raw manure on fresh produce that are intended for human consumption is discouraged due to the possible presence of human pathogens (Timmenga & Associates Inc. 2003; NOP 2006; California Leafy Green Handler Marketing Board 2010). Physically heat-treated poultry litter is used as biological soil amendment or organic fertilizer by both organic and conventional farms (Kim *et al.* 2012). To produce heat-treated poultry litter products, heat treatments, such as pelletization, pasteurization, and dry heat at higher temperatures (>65ºC for 1 h or more), are recommended to reduce or eliminate potential pathogenic microorganisms in

poultry litter while lowering the moisture content of raw materials to below 10-12% (Cox *et al.* 1986; López-Mosquera *et al.* 2008; Murray 2011). However, there is very limited research on the microbiological safety of physically heat-treated poultry litter.

Recently, our lab has carried out a series of studies on evaluating if some recommended heat treatment conditions in terms of time-temperature combination are sufficient to produce the finished products free from foodborne pathogens (Kim *et al.* 2012; Chen *et al.* 2013; Chen *et al.* 2015a; Chen *et al.* 2015b). In our previous study, the impact of desiccation adaptation of *S. enterica* during aged broiler litter stockpiling on the heat resistance during subsequent exposure to heat treatments was also investigated (Chen *et al.* 2015b). We found ca. >3-, >4-, 4-10-, and >6-fold increases in the exposure times required for reducing 5 logs of desiccation-adapted *Salmonella* cells at 70, 75, 80, and 85°C, respectively, as compared to non-adapted cells. Therefore, to validate thermal processing of poultry litter, *Salmonella* cells subjected to desiccation stress during storage should be used.

Due to biosafety concern, it is not feasible to validate thermal processing with pathogenic bacteria in poultry litter processing plants. To understand the pathogen behaviors in processing environments, suitable surrogates or indicator microorganisms with similar characteristics to pathogens are thus needed (Harris *et al.* 2013). *Enterococcus faecium* NRRL B-2354 has been used as a surrogate for *S. enterica* for validating thermal processing of almonds and carbohydrate-protein meal (Jeong *et al.* 2011; Bianchini *et al.* 2014). Almond Board of California (2007) also recommended this strain as surrogate for *S.* Enteritidis PT30 during moist-air heating of almonds.

Another approach for predicting pathogen survival during thermal processing is to monitor the behavior of indigenous microflora as indicator microorganisms. However, limited information exists on the comparison of survival behaviors of foodborne pathogens and indigenous microflora in foods or animal wastes during heat treatment. Juneja *et al.* (1997; 2003) reported the similar thermal death rate of indigenous microflora in ground beef as those of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *S. enterica*. Meanwhile, it has been recognized that enterococci can be resistant to various environmental stresses, such as high temperature and desiccation (Flahaut *et al.* 1996; Fernández *et al.* 2009). For processing biosolids, enterococci are a more representative indicator of bacterial and viral pathogens than fecal coliforms (Viau and Peccis 2009). Graham *et al.* (2009) sampled three conventional broiler chicken farms and reported enterococci population ranging from 3.00 to 7.50 log cfu g^{-1} in chicken litter during 120 d of stockpiling. In our previous study, mesophiles (incubated at 35°C) in broiler litter ranged from 6.55 to 7.80 log cfu g^{-1} during a 9-month storage (Chen *et al.* 2015b). Therefore, the populations of these indigenous microflora in poultry litter are sufficiently high for indicating the survival behavior of *S. enterica*, suggesting that they can be considered as a potential choice to validate the effectiveness of thermal processing.

Although studies have been reported using indicator microorganisms for food processing, little attention has been paid to identify indicator microorganisms for foodborne pathogens in animal wastes during thermal processing. The objective of this study was thus to select the indigenous indicator microorganisms for validating desiccation-adapted *Salmonella* reduction in physically heat-treated poultry litter.

MATERIALS AND METHODS

Sample preparation. Aged broiler litter was sourced from Cobb broiler chickens (Organic Farms, Livingston, CA). To prepare aged broiler litter, the litter inside the chicken house was removed annually followed by a partial windrow composting of 7-10 d. Afterwards, the litter was screened out of rice hulls. Composted turkey litter was obtained from Sustâne Natural Fertilizer Inc., MN. All the samples were dried under the fume hood until moisture content was reduced to <20%, and then screened to the particle size of <3 mm using a sieve (sieve pore size, 3 by 3 mm). Sufficient samples were collected for the entire study and stored in a sealed container at 4°C until use.

Analyzing physical and chemical characteristics of poultry litter

Moisture content was measured with a moisture analyzer (model IR-35, Denver Instrument, Denver, CO). Water activity (a_w) was measured with a dew-point a_w meter (Aqualab series 3TE, Decagon Devices, Pullman, WA). Ammonia content and pH value were measured based on the method described by Weatherburn (1967) and U.S. Composting Council (2002), respectively. Electrical conductivity was determined using the Orion™ VERSA STAR™ conductivity meter (Thermo Fisher Scientific Inc., Waltham, MA) based on the method described by U.S. Composting Council (2002). Duplicate litter samples were analyzed by Agricultural Service Laboratory at Clemson University for chemical characteristics, including nutrients and metals (total

concentrations, including water-soluble and water-insoluble concentrations), based on the methods recommended by U.S. Composting Council (2002).

Bacterial strain and culture conditions. *S. enterica* Senftenberg ATCC 43845 (775/W) was used for the thermal inactivation study, as our previous study revealed that *S.* Senftenberg 775/W was most heat resistant among 4 *Salmonella* strains with different serotypes tested during thermal processing of aged broiler litter (Chen *et al.* 2013). This strain was induced to rifampin resistance (100 μg ml^{-1}) using gradient plate method (Smith *et al.* 1982). *S.* Senftenberg 775/W was grown overnight at 35°C in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) containing 100 μg of rifampin ml^{-1}. The overnight cultures were washed three times with sterile 0.85% saline, and the final pelleted cells were resuspended in 0.85% saline to desired cell concentrations (ca. 10^9 cfu ml^{-1}) through adjusting the optical density at 600 nm to ca. 0.7. Afterwards, the resuspended culture was further concentrated 100 times (ca. 10^{11} cfu ml^{-1}) by centrifugation.

Desiccation adaptation. Broiler litter and turkey litter used for desiccation adaptation was exposed to greenhouse conditions for 15 d to lower the ammonia contents to 81.21±9.43 and 72.32±10.21 μg g^{-1}, respectively, in order to minimize the population reduction during desiccation adaptation (Chen *et al.* 2015a). The poultry litter with lower ammonia contents was adjusted to the desired moisture contents of 20, 30, 40, and 50% with sterile tap water. Briefly, as described by Chen *et al.* (2013), washed *S.* Senftenberg 775/W cells were added (1:10, v/w) into 100 g of poultry litter with lower ammonia content at a final concentration of ca. 10^{10} cfu g^{-1} in a container covered loosely by

aluminum foil, mixed well using a sterile blender (KitchenAid Inc., St. Joseph, MI), and incubated at room temperature. After the 24-h desiccation adaptation, poultry litter with desiccation-adapted cells was further mixed (1:100, w/w) with 500 g of poultry litter with the same moisture content using the blender for subsequent heat treatment. To enumerate indigenous enterococci and total aerobic bacteria during heat treatment, 100 g of poultry litter without adding *S.* Senftenberg 775/W was also incubated at room temperature for 24 h and then mixed with 500 g of poultry litter for subsequent experiments.

Thermal inactivation. The initial populations of *S.* Senftenberg 775/W, indigenous enterococci and total aerobic bacteria were determined right before thermal inactivation. Twenty grams of samples in triplicate were distributed evenly inside an aluminum pan (I.D. 10 cm), placed at three different locations (close to the door, center, and far away from the door) on the shelf of a controlled convectional oven (Binder Inc., Bohemia, NY), and then exposed to 75 and 85°C. Temperature was monitored using T-type thermocouples (DCC Corp., Pennsauken, NJ), with one cord kept inside the oven chamber and others inserted into litter samples at three different locations. The temperature of the oven was initially set 5°C higher than the target temperature to reduce the come-up time (time to reach the target temperature). When the interior temperature of litter sample reached the target temperature (0 h), temperature setting was readjusted to the designated temperature. Chicken litter samples were withdrawn from the oven at 0 h and every 0.5 h during holding time of 3 h to determine microbial populations. For poultry litter with 40 and 50% moisture contents, samples were collected every 0.5 h during come-up times at 85°C. Samples were transferred into a Whirl-Pak bag (Nasco,

Fort Atkinson, WI), and placed immediately in an ice-water bath to cool down the samples and minimize further cell death.

Bacterial enumeration. The surviving *Salmonella* cells were enumerated using a modified two-step overlay method to allow heat-injured cells to resuscitate, with Xylose-Lysine-Tergitol 4 agar (XLT-4; Becton, Dickinson and company, Sparks, MD) supplemented with 100 μg rifampin ml^{-1} as the selective media and tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD) as the nonselective media (Chen *et al.* 2013). Samples which were negative for *Salmonella* by direct plating method were pre-enriched in universal pre-enrichment broth (UPB; Neogen Corp., Lansing, MI) followed by a secondary enrichment in Rappaport-Vassiliadis broth (RV; Becton, Dickinson and company, Sparks, MD) supplemented with 100 μg rifampin ml^{-1}. After 24-h incubation at 42°C, enriched cultures were then plated onto XLT-4 supplemented with 100 μg rifampin ml^{-1}. The media used for total aerobic bacteria and enterococci were TSA and bile esculin agar (BEA; Becton, Dickinson and Company, Sparks, MD), respectively. Some preliminary experiments were carried out to confirm enterococci isolates from BEA agar. Colonies on BEA agar were randomly selected, and a rapid PCR assay using enterococci-specific PCR primers designed by Ke *et al.* (1999) was conducted. All isolates were confirmed to be enterococci though this method. Plates were incubated at 35°C for 24 h. The detection limits of *S.* Senftenberg 775/W, total aerobic bacteria, and enterococci by direct plating were 1.30 log cfu g^{-1}.

Statistical analysis. All experiments were conducted in two separate trials. Plate count data were converted to log cfu g^{-1} in dry weight. Differences among groups were

analyzed by least significant differences (LSD) using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA) and were considered to be significant when $P<0.05$. Linear regression analysis was performed to calculate the correlation between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci or total aerobic bacteria. An adjusted regression coefficient R^2 was used to evaluate the correlation.

RESULTS

Table 5.1 shows the chemical compositions of aged broiler litter and composted turkey litter. Organic matter, C, N, S, and Na contents, C/N, pH, EC, and ammonia content in broiler litter were much higher than those in turkey litter ($P<0.05$), while P, K, Ca, Mg, and heavy metal contents, including Zn, Cu, Mn, Fe, and Al, in turkey litter were much higher than those in broiler litter ($P<0.05$). The a_w values of broiler litter with 20, 30, 40, and 50% moisture contents were 0.808, 0.916, 0.952, and 0.974, respectively, whereas the a_w values of turkey litter with 20, 30, 40, and 50% moisture contents were 0.839, 0.917, 0.953, and 0.972, respectively. The initial populations of inoculated *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria in broiler litter and turkey litter were 7.00-7.20, 3.90-4.10, and 7.70-7.90 log cfu g^{-1}, respectively. Come-up times for heating broiler litter and turkey litter with different moisture contents at 75 and 85ºC ranged from 0.82 to 3.00 h (Table 5.2). Obviously, the higher moisture content of poultry litter required the longer come-up time.

As shown in Figure 5.1, 5.2, 5.3, and 5.4, there were reductions in microbial populations in all poultry litter samples during heat treatment at 75 and 85ºC. After both come-up times and 3-h holding times, there were significant reductions in microbial populations under various conditions (P<0.05), although the differences in populations between some sampling points within 3-h holding times were not significant (P>0.05). During come-up times, the populations of desiccation-adapted *S.* Senftenberg 775/W decreased more rapidly as compared to both types of indigenous microflora under all conditions. Microbial cells were inactivated much faster when temperature increased (P<0.05). For example, during the 0.95-h come-up time at 75ºC, population reductions of desiccation-adapted *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria in broiler litter with 20% moisture content were 3.43, 0.23, and 0.64 log cfu g^{-1}, respectively (Figure 5.1A). During the 0.88-h come-up time at 85ºC, population reductions of desiccation-adapted *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria in broiler litter with 20% moisture content were 3.79, 0.50, and 0.78 log cfu g^{-1}, respectively (Figure 5.1B).

Population reductions became higher when moisture content of poultry litter increased (P<0.05). For example, during the 0.82-h come-up time at 85ºC, population reductions of desiccation-adapted *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria in turkey litter with 20% moisture content were 4.73, 1.01, and 0.49 log cfu g^{-1}, respectively (Figure 5.1D). In comparison, during the 3.00-h come-up time at 85ºC, population reductions in turkey litter with 50% moisture content were 5.79, 2.25, and 1.44 log cfu g^{-1}, respectively (Figure 5.4D). During the 3-h holding time at 75ºC,

population reductions of desiccation-adapted *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria in turk5ey litter with 20% moisture content were 0.47, 0.09, and 0.17 log cfu g^{-1}, respectively (Figure 5.1C), while population reductions in turkey litter with 40% moisture content were 0.49, 0.35, and 0.37 log cfu g^{-1}, respectively (Figure 5.3C).

Additionally, microbial cells in broiler litter were more heat-sensitive as compared to those in turkey litter (P<0.05). For example, population reductions of desiccation-adapted *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria in turkey litter with 20% moisture content at 85ºC for 3 h were 0.27, 0.70, and 0.13 log cfu g^{-1}, respectively (Figure 5.1D). In comparison, their population reductions in broiler litter with 20% moisture content were much higher under the same treatment condition (P<0.05), which were 1.60, 1.29, and 0.42 log cfu g^{-1} for desiccation-adapted *S.* Senftenberg 775/W, indigenous enterococci, and total aerobic bacteria, respectively (Figure 5.1B).

For the linear regression analysis of inactivation data, microbial count at 0 h (when the interior temperature reached the target temperature) was chosen as the initial point. Thus the mean log reduction achieved during come-up time was not considered. When the mean log reductions of desiccation-adapted *S.* Senftenberg 775/W were compared to those of indigenous microflora by regression analysis, the linear correlation coefficients (R^2) presented in Table 5.3 and regression equations in Table 5.4 were obtained. However, microbial population data of broiler litter and turkey litter samples with 40% moisture content heat-treated at 85ºC during come-up time was used for

correlation analysis, since desiccation-adapted *S.* Senftenberg 775/W cells were not detectable by direct plating during holding time, indicating that no exact values were obtained. Additionally, desiccation-adapted *S.* Senftenberg 775/W in both poultry litter samples with 50% moisture contents at 85ºC rapidly became undetectable by direct plating but was still detectable by enrichment during come-up time. Therefore, it is impossible to compute the correlations between desiccation-adapted *S.* Senftenberg 775/W (no exact values) and indigenous microflora in both poultry litter samples with 50% moisture contents during come-up time at 85ºC.

According to our data, it is obvious that when considering the correlations between desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci, there is a high level of agreement among all combinations with the exception of turkey litter sample with ≥40% moisture content heat-treated at 85ºC. Compared to total aerobic bacteria, there were better correlations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci in broiler litter samples with 20 (R^2=0.91), 30 (R^2=0.91), 40 (R^2=0.93), and 50% (R^2=0.94) moisture contents at 75ºC, and 20 (R^2=0.98), 30 (R^2=0.87), and 40% (R^2=0.98) moisture contents at 85ºC. The mean log reductions of desiccation-adapted *S.* Senftenberg 775/W were better-correlated with those of indigenous enterococci in turkey litter samples with 20 (R^2=0.89), 30 (R^2=0.89), 40 (R^2=0.88), and 50% (R^2=0.92) moisture contents at 75ºC, and 20 (R^2=0.94) and 30% (R^2=0.83) moisture contents at 85ºC than those of total aerobic bacteria. However, total aerobic bacteria had a better correlation in turkey litter sample with 40% (R^2=0.98) moisture content at 85ºC.

DISCUSSION

As reported, foodborne pathogens can display some enhanced heat resistance after exposure to desiccation stress (Martínez *et al.* 2003; Álvarez-Ordóñez *et al.* 2008). Nevertheless, the effect of desiccation stress on heat resistance has been less studied, although it can be concluded from our recent studies that the exposure of microbial cells to desiccation can be accompanied by an increase in heat resistance (Chen *et al.* 2013; Chen *et al.* 2015a; Chen *et al.* 2015b).

Salmonella enterica can possibly be detected in poultry litter (Chen and Jiang 2014), and the populations of *Salmonella* in broiler litter and turkey litter may range from 0.6 log to 5.0 log MPN g^{-1} and from <1.0 log to >5.3 log MPN g^{-1}, respectively (Santos *et al.* 2005; Chinivasagam *et al.* 2010). Harris *et al.* (2013) stated that the inoculation level of the target microorganism in soil amendment of animal origin should reflect the upper end of the expected range, even if the isolation frequency of pathogens shed by animals is not that high. In this study, as the populations of *S.* Senftenberg 775/W at 0 h were <5.00 log cfu g^{-1} under all treatment conditions, it is reasonable to start with a higher initial population of ca. 7.80 log cfu g^{-1} for heat treatment.

It can be assumed that some robust heat-resistant *Salmonella* cells in poultry litter surviving heat treatment could potentially contaminate fresh produce during agricultural land application (Chen *et al.* 2013). Accordingly, heat-resistant *S.* Senftenberg 775/W was used in the present study to simulate the 'worst-case scenario' during thermal

processing of poultry litter. Similarly, in an early work of Liu *et al.* (1969), *S.* Senftenberg 775/W was selected as test strain when investigating the heat treatment on dry animal feeds. Ceustermans *et al.* (2007) also used *S.* Senftenberg 775/W as indicator microorganism to determine the hygienic safety of biowastes and garden wastes during composting.

Theoretically, heating unit with longer come-up time is supposed to have larger log reductions during this time period (Chen 2007a). The log reductions during come-up time are thus uniquely dependent on the individual heating unit. Therefore, to eliminate this come-up time dependency, inactivation data during holding time are widely used for downstream data analysis (Chen 2007b). When Rajan *et al.* (2006a; 2006b) and Ahn *et al.* (2007) investigated the thermal inactivation kinetics of bacterial spores, spore lethality during come-up time was not considered for model parameter estimation or curve fitting, and the spore count measured immediately after come-up time was used as the initial count. In the study of Li *et al.* (2005) on the heat resistance of *S. enterica*, *L. monocytogenes,* and *S. aureus* in high solids egg mixes, *D*-values were calculated only based on pasteurization holding time and did not reflect population reductions during come-up time. In the present study, for all treatment conditions except treatment of poultry litter samples with 40% moisture content at 85ºC, only the log reduction data during holding time were used for linear regression analysis. Nevertheless, it was observed that for poultry litter samples with 40% moisture content heat-treated at 85ºC, there were good correlations (R^2>0.88) during come-up time between *S.* Senftenberg 775/W and indigenous enterococci or total aerobic bacteria.

Clearly, the mean log reductions of *S.* Senftenberg 775/W during holding times were well-correlated with those of indigenous enterococci in broiler litter and turkey litter under all treatment conditions, with the exception of turkey litter with ≥40% moisture content heat-treated at 85°C. Therefore, this group of indigenous microflora can potentially function as indicator microorganism for *S. enterica* during thermal processing of a range of poultry litter products under different treatment conditions. This is the first report on using naturally occurring indigenous microflora as indicator microorganism for foodborne pathogen during heat treatment of animal wastes. In support of our findings, Bianchini *et al.* (2014) also reported that there was no difference between the slopes of reduction curves as a function of temperature of *S. enterica* and *E. faecium* NRRL B-2354 during extrusion of balanced carbohydrate-protein meal ($P>0.05$), suggesting that this strain could be an adequate surrogate for *S. enterica* in extrusion processes. The regression equation between log reductions of these two microorganisms can be expressed as S=E+3.01, which is similar to our results shown in Table 5.4.

Although the mean log reductions of *S.* Senftenberg 775/W were closely correlated with those of indigenous enterococci, the correlations for broiler litter and turkey litter are inconsistent. In the current study, microbial cells in turkey litter were more heat-resistant as compared to those in broiler litter ($P<0.05$). The composition of heated matrix is known to have a major influence on the thermal resistance of bacteria (Ma *et al.* 2007). As shown in Table 5.1, broiler litter and turkey litter possess different chemical characteristics, which could be attributed to the difference in poultry diet recipe and nutrient metabolism, litter management practices, or environmental source. Ammonia

content in broiler litter was much higher than that in turkey litter (P<0.05). Our previous study showed that higher ammonia content could result in weaker heat resistance of *Salmonella* cells in broiler litter (Chen *et al.* 2015b). Meanwhile, as reported by Kim *et al.* (2012), aged broiler litter used had high contents of heavy metals, which might become more soluble at higher moisture contents and lead to stronger heat resistance of *Salmonella*. Similarly, in this study, heavy metal contents in turkey litter were significantly higher as compared to those in broiler litter (P<0.05), which could be another possible explanation why microbial cells in broiler litter were more heat-sensitive than those in turkey litter. Not only is the effect of chemical composition of poultry litter significant, its physical structure may also be influential on pathogen survival during heat treatment. Therefore, it is worth emphasizing that these variables should be taken into considerations when validating thermal processing of poultry litter.

Based on our results, the correlations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous indicator microorganisms in poultry litter varied as moisture content increased from 20 to 50%. Noticeably, the mean log reductions of desiccation-adapted *S.* Senftenberg 775/W were better-correlated with those of total aerobic bacteria in turkey litter with 40% moisture content at 85ºC as compared to those of indigenous enterococci, which had a better correlation in turkey litter with 20 and 30% moisture contents at 85ºC. As moisture contents of broiler litter and turkey litter can range from 20.0 to 48.7% (in this study, 20-50% moisture contents were thus considered) (Chastain *et al.* 2001; Kelleher *et al.* 2002), the variation in correlation can possibly occur under real-world thermal processing conditions. In this study, indigenous

enterococci were substantially more heat-resistant than desiccation-adapted *S.* Senftenberg 775/W at higher temperature (85ºC) and higher moisture contents (40 and 50%) (P<0.05), as desiccation-adapted *S.* Senftenberg 775/W became undetectable by direct plating within a short time during come-up time. Similarly, as demonstrated by Sahlström *et al.* (2008), indigenous enterococci in biowaste were more heat-resistant as compared to indigenous coliforms, thermotolerant coliforms, and presumptive *E. coli*. As is well-known, only microorganisms possessing stronger heat resistance than pathogens of safety concern can be considered as suitable indicator microorganisms to avoid false negative results and ensure sufficient safety margin (Ma *et al.* 2007). Depending on the safety margin desired, poultry litter processors could use indigenous enterococci as indicator microorganism for validation of thermal processing.

It should be recognized that indigenous microflora in poultry litter may vary among batches, depending on some factors, such as its source and storage condition (Chen *et al.* 2015b). The heat resistance characteristics and populations of indigenous enterococci and total aerobic bacteria in poultry litter can thus vary from one batch to another. Hence, we acknowledge that there are some limitations in this study, especially when taking the heterogeneity of poultry litter into consideration. Our findings showed that indigenous enterococci may be used to validate the thermal processing of poultry litter under certain conditions, such as broiler litter with 20-50% and 20-40% moisture contents at 75 and 85ºC, respectively, and turkey litter with 20-50% and 20-30% moisture contents at 75 and 85ºC, respectively. The above observations were made under specific experimental conditions and thus more work is warranted to evaluate whether it

can be generalized to other poultry litter samples and treatment conditions. Further analysis on the microbial community structure of indigenous microflora as indicator microorganism in various poultry litter samples is also necessary.

In conclusion, our results demonstrated that indigenous enterococci can be used to predict the survival behavior of *S. enterica* during thermal processing of poultry litter under current litter processing conditions, without introducing pathogens into the industrial environment. Moreover, thermal inactivation data from this study will assist poultry litter processors in designing some effective processing time-temperature regimes to ensure the microbiological safety of physically heat-treated poultry litter.

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Table 5.1 Chemical characteristics of aged broiler litter and composted turkey litter*

Poultry litter	Nutrient (%)									
	OM†	C	N	C/N	P	K	Ca	Mg	S	Na
Broiler litter	67.10± 1.41a	34.26± 0.36a	3.75± 0.01a	9.13± 0.13a	2.77± 0.13b	2.89± 0.14b	2.56± 0.10b	0.56± 0.02b	1.06± 0.04a	0.91± 0.04a
Turkey litter	41.40± 2.69b	25.37± 0.46b	2.97± 0.05b	8.56± 0.01b	5.43± 0.18a	4.06± 0.19a	5.31± 0.29a	1.10± 0.07a	0.85± 0.03b	0.68± 0.03b

	Heavy metal (μg g^{-1})					pH	EC‡ (ms cm^{-1})	NH_4-N (μg g^{-1})
	Zn	Cu	Mn	Fe	Al			
Broiler litter	439.00± 21.21b	250.50± 14.85b	472.00± 21.21b	3103.00± 104.65b	2710.00± 11.31b	8.70± 0.00a	14.84± 0.22a	820.35± 67.37a
Turkey litter	488.50± 23.33a	626.00± 35.36a	592.00± 22.63a	6880.00± 429.21a	4526.50± 246.78a	8.00± 0.00b	13.31± 0.06b	591.26± 25.61b

*Data are expressed as means±SD of two samples. Means with different letters in the same column are significantly different (P<0.05). The values of nutrients and metals are all calculated based on dry-weight.

†OM, organic matter.

‡Electrical conductivity.

Table 5.2 Come-up times for aged broiler litter and composted turkey litter at 75 and 85ºC

Moisture content (%)	Temperature (°C)	Come-up time (h) with poultry litter	
		Aged broiler litter	Composted turkey litter
20	75	0.95	0.85
	85	0.88	0.82
30	75	1.38	1.52
	85	1.27	1.35
40	75	2.05	2.07
	85	1.70	1.77
50	75	2.48	3.12
	85	2.00	3.00

Table 5.3 Regression correlations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci or total aerobic bacteria in aged broiler litter and composted turkey litter with 20, 30, 40, and 50% moisture contents at 75 and 85°C

Moisture content (%)	Temperature (°C)	Microorganism	Regression correlation (R^2) with poultry litter	
			Aged broiler litter	Composted turkey litter
20	75	Enterococci	0.91±0.03a*	0.89±0.02a
		Total aerobic bacteria	0.84±0.02b	0.81±0.03b
	85	Enterococci	0.98±0.02a	0.94±0.05a
		Total aerobic bacteria	0.91±0.03b	0.63±0.07b
30	75	Enterococci	0.91±0.04a	0.89±0.02a
		Total aerobic bacteria	0.75±0.05b	0.87±0.02a
	85	Enterococci	0.87±0.04a	0.83±0.06a
		Total aerobic bacteria	0.80±0.01b	0.45±0.05b
40	75	Enterococci	0.93±0.06a	0.88±0.02a
		Total aerobic bacteria	0.62±0.05b	0.83±0.02b
	85	Enterococci	0.98±0.03a†	0.88±0.03b†
		Total aerobic bacteria	0.91±0.03b†	0.98±0.04a†
50	75	Enterococci	0.94±0.02a	0.92±0.02a
		Total aerobic bacteria	0.86±0.03b	0.90±0.01a
	85	Enterococci	N.A.‡	N.A.
		Total aerobic bacteria	N.A.	N.A.

*Data are expressed as means±SD of two trials. For each temperature, means with different letters in the same column are significantly different (P<0.05).
†Population data during come-up time was used.
‡N.A., not applicable.

Table 5.4 Regression equations between mean log reductions of desiccation-adapted *S.* Senftenberg 775/W and indigenous enterococci or total aerobic bacteria in aged broiler litter and composted turkey litter with 20, 30, 40, and 50% moisture contents at 75 and 85°C

Moisture content (%)	Temperature (°C)	Regression equation with poultry litter	
		Aged broiler litter	Composted turkey litter
20	75	S[*]=2.63E-1.68	S=2.71E+0.05
	85	S=1.04E+0.31	S=2.38E
30	75	S=1.18E-1.04	S=2.74E-0.29
	85	S=0.77E+0.35	S=1.34E+0.73
40	75	S=2.08E+0.98	S=1.30E-0.29
	85	S=2.89E+1.41[†]	S=1.20B[a]+4.58[†]
50	75	S=0.06E-0.06	S=0.31E+0.02
	85	N.A.[‡]	N.A.

[*]S, *S.* Senftenberg 775/W; E, enterococci; B, total aerobic bacteria.
[†]Population data during come-up time was used.
[‡]N.A., not applicable

Figure 5.1 Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 20% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown.

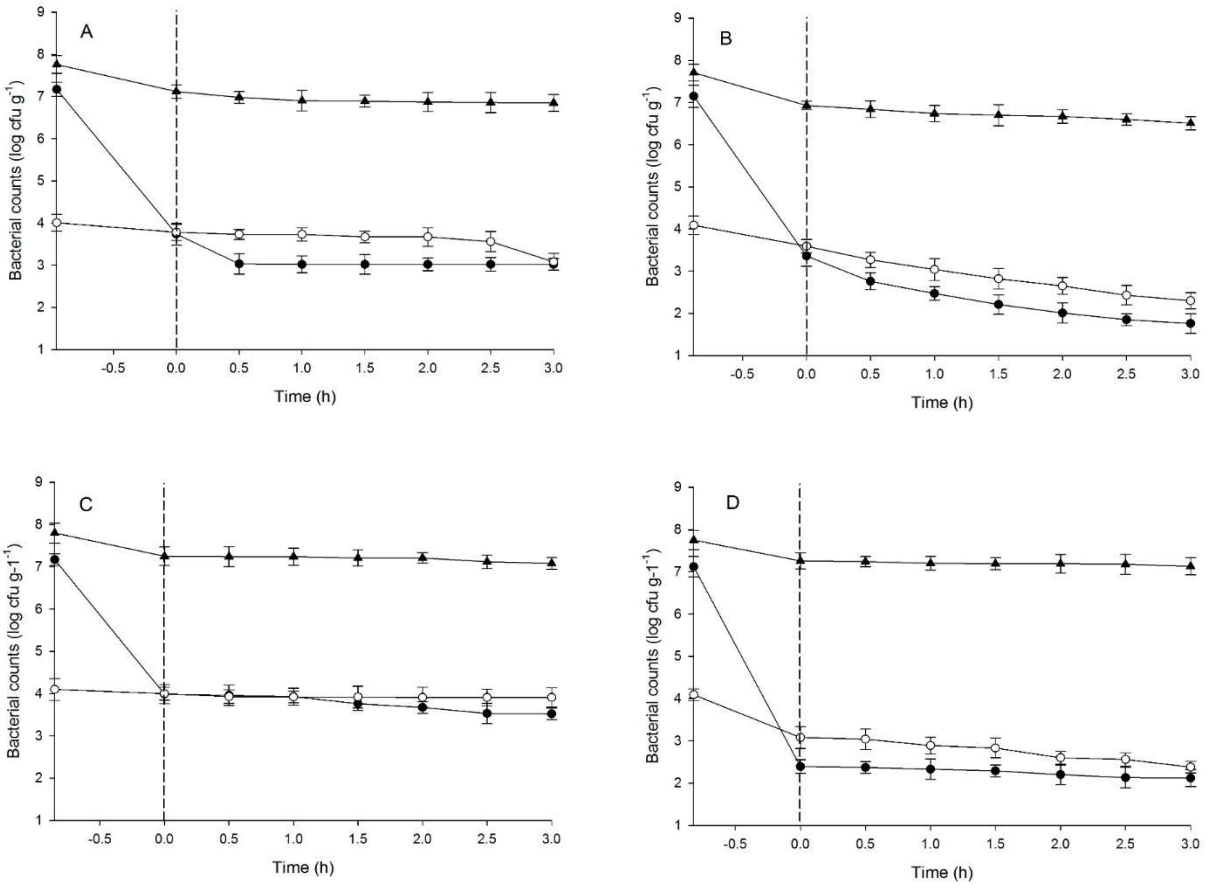


Figure 5.2 Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 30% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown.

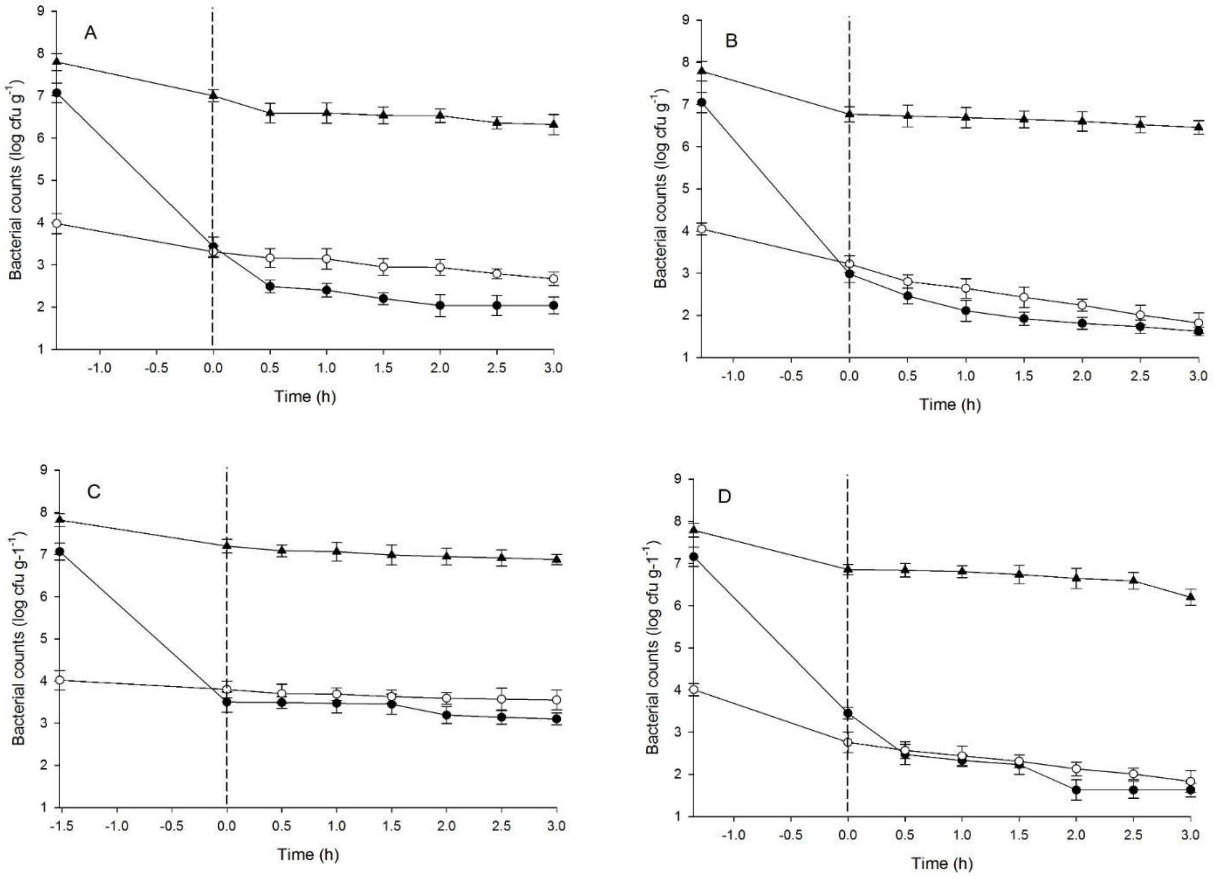


Figure 5.3 Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 40% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown.

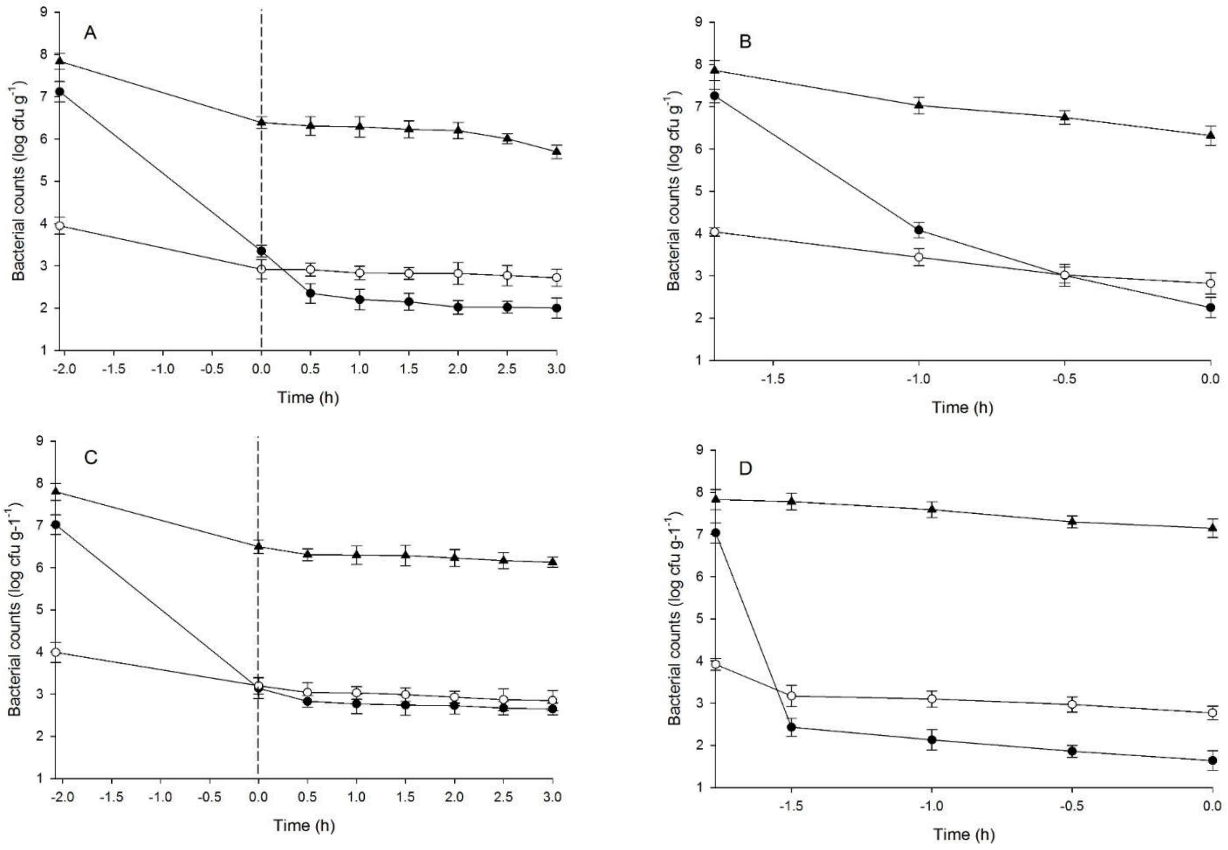
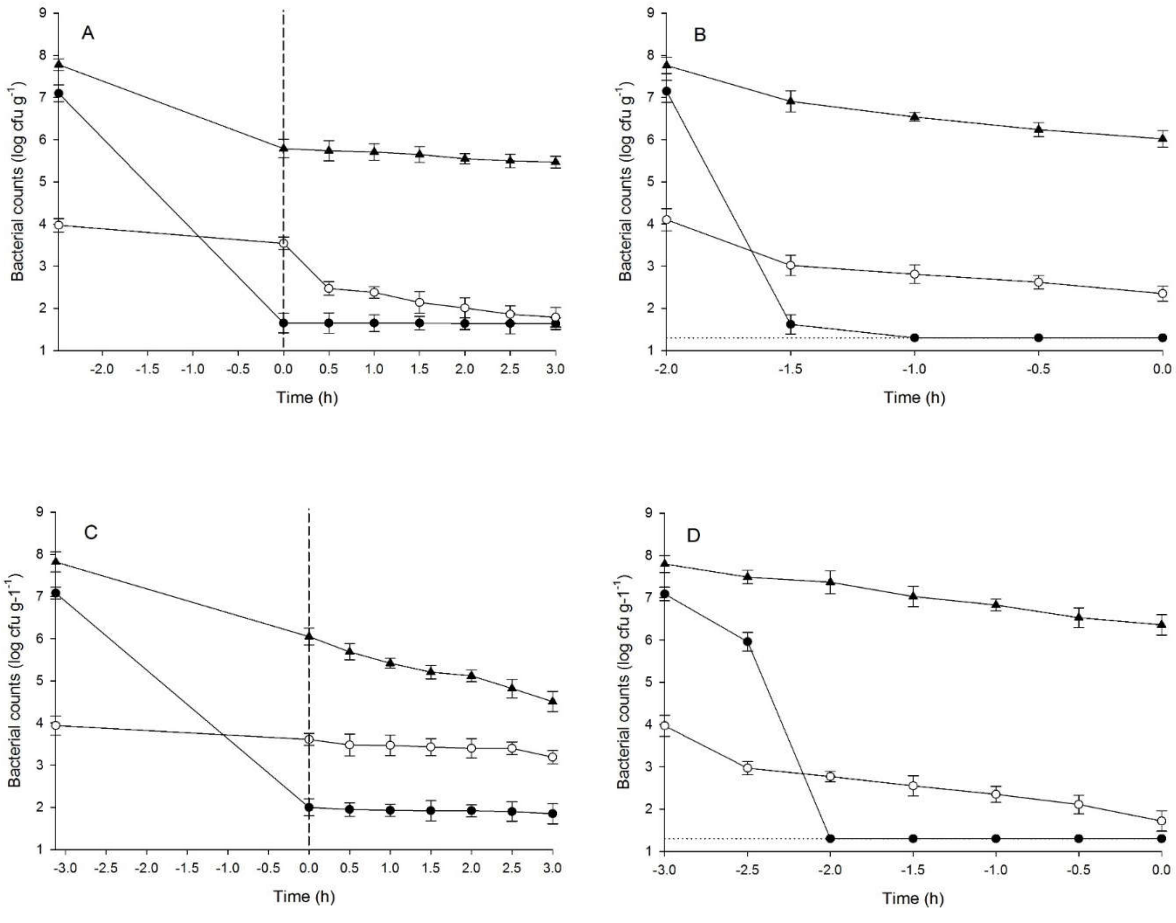


Figure 5.4 Survival of desiccation-adapted *S.* Senftenberg 775/W (solid circle), indigenous enterococci (hollow circle), and total aerobic bacteria (solid triangle) in aged broiler litter and composted turkey litter with 50% moisture content at 75 and 85°C. A: aged broiler litter at 75°C; B: aged broiler litter at 85°C; C: composted turkey litter at 75°C; D: composted turkey litter at 85°C. Inactivation curves during come-up times (on the left of the vertical dashed line) and during holding times (on the right of the vertical dashed line) are shown. The horizontal dotted line indicates that *Salmonella* was detectable only by enrichment (detection limit by direct plating: 1.30 log cfu g^{-1}).



CHAPTER SIX

THERMAL RESISTANCE AND GENE EXPRESSION OF BOTH DESICCATION-ADAPTED AND REHYDRATED *SALMONELLA ENTERICA* TYPHIMURIUM CELLS IN AGED BROILER LITTER

ABSTRACT

The objective of this study was to investigate the thermal resistance and gene expression of both desiccation-adapted and rehydrated *Salmonella enterica* Typhimurium cells in aged broiler litter. *S.* Typhimurium was desiccation-adapted in aged broiler litter with 20% moisture content (a_w: 0.81) for 1, 2, 3, 12, or 24 h at room temperature and then rehydrated for 3 h. As analyzed by quantitative real-time reverse transcriptase PCR (qRT-PCR), *rpoS*, *proV*, *dnaK*, and *grpE* genes were up-regulated ($P<0.05$) under desiccation stress and could be induced after 1 h but less than 2 h. Following rehydration, fold changes of these four genes became significantly lower ($P<0.05$). Desiccation-adapted Δ*rpoS* mutant was less heat-resistant at 75°C than desiccation-adapted wild type ($P<0.05$), whereas there were no differences in heat resistance between desiccation-adapted mutants in two non-regulated genes (*otsA* and *PagfD*) and desiccation-adapted wild type ($P>0.05$). Survival characteristics of desiccation-adapted *ΔPagfD* (rdar morphotype) and Δ*agfD* (saw morphotype) mutants were similar ($P>0.05$). The trehalose synthesis in desiccation-adapted wild type was not induced as compared to non-adapted cells ($P>0.05$). Our results demonstrated the importance of *rpoS*, *proV*, *dnaK*, and *grpE* genes in the desiccation survival of *S.* Typhimurium. By using a *ΔrpoS* mutant, we found that *rpoS* gene was involved in the cross-protection of desiccation-adapted *S.* Typhimurium against high temperature, while trehalose synthesis or rdar morphology did

not play a significant role in this phenomenon. In summary, *S.* Typhimurium could respond rapidly to the low-a_w condition in aged broiler litter while developing the cross-protection against high temperature, but this process could be reversed upon rehydration.

INTRODUCTION

Poultry litter has been widely used as biological soil amendments for growing fresh produce; however, there are many species of human pathogens, such as *Salmonella*, that may potentially present in poultry litter or inadequately composted poultry litter (1). Erickson et al. (2) found that *S.* Typhimurium was still detectable in sub-surface samples after 14 days in static composting piles composed of chicken litter and peanut hulls. These pathogens may possibly contaminate fresh produce after agricultural land application of poultry litter, as a small population of pathogens in animal wastes may survive and persist for an extended period of time (3). Islam et al. (4) reported that *S.* Typhimurium could persist for over 200 days in soil samples amended with poultry litter-based composts.

Physical heat treatment (heat-drying after composting or without composting) is considered to be one of the effective techniques to eliminate human pathogens from animal wastes (5). However, exposure of bacterial cells to one sub-lethal stress can produce the cross-tolerance to multiple stresses (6). Some microbial cells may thus become acclimatized to the stressful environments in animal wastes, which can cross-protect them against subsequent high temperature. Actually, our previous studies have

already demonstrated that desiccation-adapted *S. enterica* in aged broiler litter displayed enhanced thermal resistance than non-adapted cells (7, 8). We observed that ca. >3-, >4-, 4-10-, and >6-fold increases in the exposure times were required for reducing 5 logs of desiccation-adapted *S. enterica* at 70, 75, 80, and 85°C, respectively, as compared to non-adapted cells. Gruzdev et al. (9) also reported that desiccated *S. enterica* cells exhibited strong tolerance to dry heat at 60°C, without any significant population reduction in 1 h, in comparison to a 3-log reduction in the number of non-desiccated cells under identical conditions. Nonetheless, comparatively little is known about the underlying mechanisms of the cross-protection of desiccation-adapted *Salmonella* against subsequent high temperature.

There have been some published studies on the gene expression of *S. enterica* under various low-a_w conditions (10, 11, 12, 13, 14, 15). A diverse range of responses in *Salmonella* can be induced in response to desiccation, depending on the experimental condition (e.g. a_w and the method used to create a low-a_w environment) and strain tested. Genes that have been reported to be up-regulated upon desiccation in these studies are involved in some biological processes, such as general stress response, trehalose synthesis, osmoprotectant production, fatty acid metabolism, and heat shock response.

Extracellular components, such as thin aggregative fimbriae (tafi), are required for the long-term persistence of some microorganisms under desiccation conditions (16). *S. enterica* Typhimurium possesses the multicellular rdar (red, dry, and rough) morphotype which is regulated by *agfD* promoter (17). Environmental stresses, such as desiccation and starvation, can trigger the expression of rdar (red, dry, and rough) morphology genes

in *S.* Typhimurium (17). The presence of rdar morphology can improve the colonization of *Salmonella* on and in fresh produce (18) and facilitate the dispersal of the pathogen in post-rain aerosols (19). Both rdar and non-rdar morphotypes, including saw (smooth and white) morphotype, can possibly be isolated from various sources, such as poultry, produce, and clinical samples (20). If poultry litter used as biological soil amendments contains the rdar morphotype, the pathogen may possibly transfer to fresh produce after agricultural land application. In spite of the scientific importance and practical implications of rdar morphology, its thermal resistance remains poorly understood. Scher et al. (21) reported that pellicle cells (rdar morphotype) were significantly more resistant to sodium hypochlorite; however, the stress management of pellicle cells in response to heat or low pH was not enhanced compared to that of planktonic cells.

As far as we know, there is no available information about the thermal resistance of desiccation-adapted microorganisms upon rehydration. In an effort to fill this knowledge gap, we thus investigated the thermal resistance and gene expression of both desiccation-adapted and rehydrated *S.* Typhimurium in aged broiler litter. Elucidating the response of *S.* Typhimurium to desiccation stress in aged broiler litter can provide insight into how this pathogen can survive under low-a_w conditions and how the exposure to this condition influences its heat resistance.

MATERIALS AND METHODS

Sample preparation. Aged broiler litter was sourced from Cobb broiler chickens (Organic Farms, Livingston, CA). To prepare aged broiler litter, the litter was removed from chicken house followed by a partial windrow composting of 7-10 d and then screened out of rice hulls. Aged broiler litter was dried under fume hood until moisture content was reduced to <20%, and then screened to the particle size of <3 mm using a sieve (sieve pore size, 3 by 3 mm). Sufficient samples were collected for all experiments and stored in a sealed container at 4°C.

Analyzing physical and chemical characteristics of poultry litter. Moisture content was measured with a moisture analyzer (model IR-35, Denver Instrument, Denver, CO). Water activity (a_w) was measured with a dew-point a_w meter (Aqualab series 3TE, Decagon Devices, Pullman, WA). Ammonia content and pH value were measured based on the method described by Weatherburn (22) and U.S. Composting Council (23), respectively.

Bacterial strains and culture preparation. *Salmonella enterica* Typhimurium ATCC 14028 wild type, IB43 (*rpoS*::Tn10dCm), and XF373 (*otsA*::MudJ) were kindly provided by Dr. Ferric C. Fang (University of Washington, Seattle, WA), whereas *S.* Typhimurium MAE 110 (*PagfD*1; rdar: aggregate/multicellular phenotype) and MAE 119 (Δ*agfD*101; saw: smooth colony morphology) were obtained from Dr. Ariena H.C. van Bruggen (University of Florida, Gainesville, FL). MAE 110 constantly possesses the rdar morphology, whereas MAE 119 (saw morphotype) has completely lost the rdar morphology. All mutants used in this study were derived from *S.* Typhimurium ATCC 14028 wild type. *S.* Typhimurium strains were grown overnight at 35°C in tryptic soy

broth (TSB; Becton, Dickinson and Company, Sparks, MD). The overnight cultures were washed three times with sterile 0.85% saline, and the final pelleted cells were resuspended in 0.85% saline to obtain the desired cell concentrations based on the optical density at 600 nm.

Desiccation adaptation and rehydration of *S.* Typhimurium. As described by Chen et al. (7), washed *S.* Typhimurium wild type cultures were mixed (1:10, v/w) with 100 g of aged broiler litter with lower ammonia content (72.66 μg g^{-1}) at a final concentration of ca. 10 log cfu g^{-1} using a sterile blender (KitchenAid Inc., St. Joseph, MI). The moisture content of the inoculated litter was then adjusted to 20% (a_w: 0.81) with sterile tap water and incubated in a sterile container covered loosely by aluminum foil at room temperature for 1-, 2-, 3-, 12-, and 24-h desiccation adaptations. *Salmonella* cells added into aged broiler litter and immediately used for heat treatment (0-h desiccation adaptation) were served as control (non-adapted cells).

To perform rehydration after 3-, 12-, and 24-h desiccation adaptations, the inoculated litter (10 g) was mixed with 30 ml of sterile water in a Whirl-Pak bag (Nasco, Fort Atkinson, WI) (12) and homogenized using a Seward 400 Circulator Lab Blender (Seward Ltd., Worthing, West Sussex, UK). The homogenate was kept at room temperature for 3 h.

Thermal inactivation of desiccation-adapted and rehydrated *S.* Typhimurium. To prepare desiccation-adapted cells as inoculum in aged broiler litter for thermal inactivation, 10 g of litter sample was homogenized with 30 ml of sterile water right before heat treatment, allowing its initial population to be consistent with that of

rehydrated cells. With respect to the inoculation of aged broiler litter, homogenate with desiccation-adapted or rehydrated cells was centrifuged at 1,500 rpm for 1 min to remove large litter particles. The supernatant was then collected and mixed (1:100, v/w) with 500 g of aged broiler litter. The moisture content of the inoculated litter was then adjusted to 20% with sterile tap water for subsequent heat treatment (ca. 7 log cfu g^{-1}).

After mixing with desiccation-adapted or rehydrated cells, 20 g of litter samples in triplicate were distributed evenly into an aluminum pan (I.D. 10 cm), placed at three different locations (close to the door, center, and far away from the door) on the shelf of a controlled convectional oven (Binder Inc., Bohemia, NY), and then exposed to dry heat at 75°C for up to 2 h. Temperature was continuously monitored with T-type thermocouples (DCC Corp., Pennsauken, NJ), with one cord inside the oven chamber and others in litter samples. The temperature of the oven was initially set at 80°C to reduce the come-up time (0.87 h). When the interior temperature of litter sample reached the target temperature, temperature setting was readjusted to 75°C. Chicken litter samples were withdrawn from the oven every 0.5 h to determine *Salmonella* population. Samples were transferred into the Whirl-Pak bag, and placed immediately in an ice-water bath to cool down the samples.

Bacterial enumeration. *Salmonella* cells were counted using a modified two-step overlay method with Xylose-Lysine-Tergitol 4 agar (XLT-4; Becton, Dickinson and company, Sparks, MD) as the selective media while tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD) as the nonselective media to allow heat-injured cells to resuscitate (7). Samples negative for *Salmonella* by direct plating method

were pre-enriched in universal pre-enrichment broth (UPB; Neogen Corp., Lansing, MI) followed by a secondary enrichment in Rappaport-Vassiliadis broth (RV; Becton, Dickinson and company, Sparks, MD). After 24-h incubation at 42°C, enriched cultures were plated onto XLT-4. The detection limits of direct plating and enrichment were 1.30 and 1.00 log cfu g^{-1}, respectively.

Gene expression study on desiccation-adapted and rehydrated *S.* Typhimurium. For gene expression study, in order to extract high-quality RNA from *S.* Typhimurium, autoclaved aged broiler litter (dry cycle at 121°C for 15 min) was used for desiccation adaptation to reduce the interference from background microflora (24). And desiccation adaptations and rehydration were conducted as aforementioned.

After desiccation adaptation or rehydration, the inoculated litter (10 g) was mixed with 50 ml of PBS in the Whirl-Pak bag and homogenized with the Seward 400 Circulator Lab Blender. The mixture was centrifuged at 1,500 rpm for 1 min to remove large particles. *S.* Typhimurium cells were then separated using Dynabeads anti-*Salmonella* (Thermo Fisher Scientific Inc., Asheville, NC) as described by Singh and Jiang (24). Afterwards, the cells attached to Dynabeads were washed twice in PBS and 2 volumes of RNAProtect Bacteria Reagent (Qiagen, Valencia, CA) were then added into 1 volume of PBS with beads for stabilization of RNA in *S.* Typhimurium.

Afterwards, RNA of *S.* Typhimurium (ca. 7 log cfu ml^{-1}) was extracted using a RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. Contaminating DNA was removed with a TURBO DNA-free Kit (Thermo Fisher Scientific Inc.). A NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.) was used to determine the

RNA concentration and purity (A_{260}/A_{280}). Overall RNA integrity was analyzed by gel electrophoresis in a 1.2% non-denaturing agarose gel in Tris-acetate buffer at 60 V for 1 h. RNA was heated at 70°C for 1 min and chilled on ice to denature the secondary structures before loading on the gel. The gel was stained in ethidium bromide solution and visualized with a Gel Doc 1000 system (Bio-Rad Laboratories, Inc., Hercules, CA).

Desiccation-associated genes were selected based on published literature (10, 11, 12, 13, 14, 15). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed in a Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany) using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Thermo Fisher Scientific Inc.). Primer sequences and qRT-PCR procedures (temperatures and incubation times) for twelve desiccation-associated genes and a reference gene were based on the references shown in Table 6.1. The PCR reactions were performed with RNA extracts from two independent trials, each with three replicates. The Pfaffl method was used to calculate the relative expression (fold change) of gene targets (34). Primer efficiency was determined by ten-fold serial dilution of the extracted RNA and calculated as described by Pfaffl (34). Gene expression was normalized against 16S rRNA as the reference gene. Differentially expressed genes were defined as significantly up- or down-regulated ≥2 folds between desiccation-adapted or rehydrated cells and non-adapted cells ($P<0.05$) (15).

Mutation study. The survival data of *S.* Typhimurium IB43, XF373, MAE 110, and MAE 119 in aged broiler litter with 20% moisture content at 75°C were compared

with those of wild type by direct plating and enrichment methods. Desiccation adaptation and thermal inactivation were carried out as described above.

Trehalose content determination. Desiccation-adapted cells were separated using Dynabeads as described above. Cells attached to Dynabeads were suspended in 5 ml of PBS and disrupted by subjecting to a vortex for 5 min in the presence of 5 g of disruptor beads with diameter of 0.1 mm (Electron Microscopy Sciences, Hatfield, PA). Cell debris, disruptor beads, and Dynabeads were then removed by centrifuging at 5,000 rpm for 10 min. The supernatant was used for subsequent determination of trehalose content.

Trehalose content was measured using an enzymatic colorimetric assay by converting trehalose to glucose with trehalase and then determining the glucose content (35, 36). Briefly, 50 µl of extract was incubated at 37°C for 6 h with 150 µl of 0.2 mol l^{-1} sodium acetate buffer (pH 5.5) and 50 µl of 0.3 U ml^{-1} trehalase. Trehalose content was calculated from the liberated glucose which was quantified using a Glucose Assay Kit (Abnova Corporation, Taipei, Taiwan). Trehalose standard solutions were used to generate a trehalose standard curve, with concentrations ranging from 0 to 10 nmol of trehalose (Sigma-Aldrich Corp., St. Louis, MO) per reaction. To nullify the interfering effect of glucose from other sources, background glucose content in the extract was determined with a parallel control containing above reaction components in which trehalase was replaced with distilled water, and was then subtracted from the total glucose content to obtain the net glucose content (37). Trehalose content was expressed as trehalose weight per total water-soluble protein weight (nmol trehalose mg $protein^{-1}$).

Protein content was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.).

Statistical analysis. All results were obtained from two independent trials. Plate count data were converted to log cfu g^{-1} in dry weight. SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA) was applied for data analysis. Analysis of variance (ANOVA), followed by the least significant differences (LSD) test, was performed to determine whether significant differences ($P<0.05$) existed among different groups.

RESULTS

As shown in Table 6.2, the populations of 3-, 12-, and 24-h desiccation-adapted *S.* Typhimurium wild type, as well as non-adapted cells, in aged broiler litter decreased during heat treatment at 75°C. However, desiccation-adapted wild type survived much longer as compared to non-adapted cells ($P<0.05$). Non-adapted cells were detectable only by enrichment (<1.30 log cfu g^{-1}) after 1.5 h, while desiccation-adapted wild type could be detected by direct plating throughout the entire treatment. And the populations of desiccation-adapted wild type were much higher than those of non-adapted cells ($P<0.05$). For example, after 1-h heat treatment, the population of non-adapted cells decreased rapidly to 2.14 log cfu g^{-1}, but there were still nearly 4 log cfu g^{-1} of desiccation-adapted wild type surviving in aged broiler litter. A 5-log reduction in the population of desiccation-adapted wild type required >2 h of heat exposure. As a comparison, a 5-log reduction in the population of non-adapted cells could be achieved

only within 1.5 h. Nevertheless, there were no significant differences in microbial population among wild type desiccation-adapted for 3, 12, and 24 h ($P>0.05$), suggesting that the heat resistance of desiccation-adapted cells were not significantly enhanced when desiccation duration was extended from 3 to 24 h.

When the effect of rehydration on the heat resistance of desiccation-adapted wild type in aged broiler litter was investigated, it was found that upon rehydration, desiccation-adapted cells in aged broiler litter became less heat-resistant. After 1-h heat treatment, the numbers of surviving desiccation-adapted cells were 1-2 log cfu g^{-1} higher than those of 3-h rehydrated cells ($P<0.05$). Moreover, during heat treatment, the populations of wild type desiccation-adapted for 3, 12, and 24 h following rehydration were similar ($P>0.05$), but were all higher than non-adapted wild type ($P<0.05$). For non-adapted cells, 3-h rehydration could not result in any change in its heat resistance ($P>0.05$).

The expressions of some desiccation-associated genes in *S.* Typhimurium wild type were studied. Four genes in *S.* Typhimurium, including *rpoS*, *proV*, *dnaK*, and *grpE*, were identified to be significantly up-regulated (>2 folds) upon 3-, 12-, and 24-h desiccation adaptations ($P<0.05$) (Figure 6.1A-C). The highest up-regulations were observed in *rpoS* and *dnaK* genes in a range of 32 to 40 folds, while *proV* and *grpE* genes were up-regulated from 2 to 9 folds. Eight other genes, including *otsA*, *otsB*, *agfD*, *kdpA*, *fadA*, *cspA*, *sigDE*, and *dps*, were not differentially expressed ($P>0.05$). There were no significant differences in fold changes of selected genes among *S.* Typhimurium desiccation-adapted for 3, 12, and 24 h ($P>0.05$). After 3-h rehydration, fold changes of

all significantly up-regulated genes became much lower ($P<0.05$). Some up-regulated genes even became non-differentially expressed (<2 folds) and returned to normal transcriptional levels post-rehydration. For example, in response to rehydration, fold changes of *rpoS* and *proV* genes in 24-h desiccation-adapted cells decreased from +37.6 to -1.0 and from +8.6 to +1.3, respectively (Figure 6.1C). Genes that were non-differentially expressed after 3-, 12-, and 24-h desiccation adaptations were still non-regulated following rehydration ($P>0.05$).

To further study if *S.* Typhimurium could respond to the desiccation stress in aged broiler litter within an even shorter period of time (≤2 h), fold changes of four differentially expressed genes (*rpoS*, *proV*, *dnaK*, and *grpE*) were thus studied upon 1- and 2-h desiccation adaptations. As shown in Figure 6.2, after 1-h desiccation adaptation, *rpoS*, *proV*, *dnaK*, and *grpE* genes were non-differentially expressed (<2 folds). However, when desiccation duration was prolonged to 2 h, these genes were significantly up-regulated 25.5-, 5.8-, 20.6-, and 2.4-fold, respectively ($P<0.05$). Fold changes of *proV* and *dnaK* genes after 2-h desiccation adaptation were similar to those after 3-, 12-, and 24-h desiccation adaptations ($P>0.05$), while fold changes of *rpoS* and *grpE* genes after 2-h desiccation adaptation were still lower as compared to those after 3-, 12-, and 24-h desiccation adaptations ($P<0.05$).

To explore if the up-regulated genes were involved in the cross-protection against subsequent high temperature, one significantly up-regulated gene, *rpoS*, was selected for our mutation study. As controls, two non-regulated genes, including *otsA* and *agfD*, were also selected. As shown in Table 6.2, the populations of desiccation-adapted and non-

adapted IB43 (Δ*rpoS* mutant) were similar throughout the entire heat treatment (P>0.05), and they were not detectable by enrichment only after 1 h, indicating that desiccation adaptation of IB43 could not lead to an increase in its heat resistance. Additionally, desiccation-adapted IB43 survived much shorter as compared to desiccation-adapted wild type (P<0.05). During 2-h heat treatment, the populations of non-adapted Δ*rpoS* mutant were significantly lower than those of non-adapted wild type (P<0.05), as non-adapted wild type could still be detected by enrichment after 2 h. In comparison, non-adapted Δ*rpoS* mutant was not detectable by enrichment only after 1 h, suggesting that the mutation in *rpoS* could also cause a reduction in the heat resistance of *S.* Typhimurium.

During heat treatment, the populations of desiccation-adapted and non-adapted XF373 (Δ*otsA* mutant) were similar to those of desiccation-adapted and non-adapted wild type (P>0.05), respectively (Table 6.1). The rdar morphotype didn't possess an increased heat resistance, as the populations of desiccation-adapted and non-adapted MAE 110 (*ΔPagfD* mutant) or MAE 119 (Δ*agfD* mutant) were not significantly different from those of desiccation-adapted and non-adapted wild type (P>0.05), respectively. And the survival data of *ΔPagfD* (rdar morphotype) and Δ*agfD* (saw morphotype) mutants were also similar throughout the whole treatment (P>0.05). Hence, *otsA* and *agfD* genes could not contribute to the cross-tolerance of desiccation-adapted *S.* Typhimurium to heat treatment.

The trehalose contents in *S.* Typhimurium wild type after 3-, 12-, and 24-h desiccation adaptations were less than 1.0 mmol mg protein^{-1} (Figure 6.3), and there were no significant differences between desiccation-adapted and non-adapted cells (P>0.05).

Therefore, trehalose was not substantially synthesized during 3-, 12-, or 24-h desiccation adaptation.

DISCUSSION

The stress responses in bacteria are controlled by master regulators, which include alternative sigma factors, such as RpoS (38). RpoS integrates multiple signals (e.g. the general stress response regulators) and regulates the expressions of over 50 genes involved in the responses to various stresses. van Hoek et al. (39) reported that a fully functional RpoS system is an advantage for the long-term survival of *Escherichia coli* O157 in the manure-amended soil environment. It is generally believed that these response pathways extensively overlap and bacteria exposed to one sub-lethal stress may thus develop cross-protection against other stresses (40). In the current study, we observed that desiccation-adapted *S.* Typhimurium in aged broiler litter could develop cross-tolerance to high temperature, which is consistent with our previous results (7, 8). Cross-protection usually occurs when an initial sub-lethal stress enhances the resistance to multiple subsequent stresses (14). This phenomenon is of particular concern in poultry litter processing industry, where poultry litter commonly undergo a series of interventions (e.g. composting and long-term stockpiling) in tandem to reduce the human pathogen load. In order to gain further insight into the mechanisms underlying, we have extended our study to identify some differentially-expressed genes in desiccation-adapted *S.* Typhimurium in aged broiler litter through transcriptomic analysis.

Genes that were up-regulated under desiccation stress. According to our data, the desiccation stress in aged broiler litter resulted in considerable up-regulations of four genes in *S.* Typhimurium, including *rpoS* (Regulator of the general stress response), *proV* (Osmoprotectant transporters), *dnaK* (Chaperone protein), and *grpE* (Heat shock protein).

In *Salmonella*, *rpoS* gene encodes an alternative sigma factor (σ^S/RpoS) that initiates the transcriptions of a series of genes and acts as a master regulator required for survival under harsh conditions (41, 42). In the current study, in agreement with the known role of *rpoS* gene in the general stress response, *rpoS* gene was found to be significantly up-regulated following desiccation in aged broiler litter ($P<0.05$). Similarly, Stasic et al. (43) observed that *E. coli* O157:H7 wild type survived much longer (>28 days) in sterile bovine feces (a_w: <0.50) than a Δ*rpoS* mutant (21 days). We also found that attenuated expression of *rpoS* gene in Δ*rpoS* mutant could also cause significantly reduced thermal resistance in aged broiler litter ($P<0.05$). As expected, desiccation adaptation of Δ*rpoS* mutant could not cross-protect this mutant against subsequent heat treatment, suggesting that *rpoS* gene is specifically required for the cross-protection development. Generally, our findings are concurrent with the idea that bacteria evoke a general stress response upon exposure to some environmental stresses (44).

When bacterial cells are exposed to low-a_w environments, such as aged broiler litter, the water activity in the cell is lowered. To combat the loss of water, they must balance the osmolarity of their internal cell composition with that of the external environment (45). During the process of osmoregulation, bacteria accumulate some osmoprotective low-molecular-weight compatible solutes known as osmoprotectants (e.g.

glycine betaine), which can concentrate to high levels within the bacterial cell without affecting enzyme function (46). ProU is known as one of the main transport systems for glycine betaine in *Salmonella* (47). ProV, encoded by *proV* gene, is a protein partially associated with the cytoplasmic membrane and serves the energy-coupling function in the ProU transport system (48). The up-regulation of *proV* gene in *Salmonella* has previously been documented in some studies focusing on the transcriptomic changes occurring during desiccation. Li et al. (15) reported that after a 2-h desiccation in a desiccator (a_w: 0.11), *proV* gene in *S.* Tennessee and *S.* Typhimurium LT2 was significantly induced by 12.5- and 14.9-fold, respectively. In the study by Finn et al. (12), *proV* gene was among the most highly up-regulated genes following a 4-h desiccation on stainless steel coupon. Consistent with above previous findings, we also observed a dramatic increase in the expression of *proV* gene in desiccation-adapted *S.* Typhimurium in aged broiler litter. We thus further emphasized the importance of *proV* gene for the survival of *Salmonella* in low-a_w environments.

Another gene induced during desiccation in aged broiler litter was *dnaK* gene, which encodes chaperone protein (Hsp70) that helps stabilize other proteins during heat shock (49). It can thus be hypothesized that the up-regulation of some heat-tolerant genes under pre-adaptive desiccation stress could potentially confer resistance to subsequent lethal heat stress. Evidence from published literature also supports the assumption that *dnaK* gene can be involved in the survival of *Salmonella* in response to desiccation stress. Gruzdev et al. (14) reported an induction of *dnaK* gene in *S.* Typhimurium dehydrated in petri dish for 22 h. When Deng et al. (11) studied the transcriptomic response of *S.*

Enteritidis to desiccation in peanut oil (a_w: 0.30) for 72, 216, and 528 h, the transcription of *dnaK* gene was observed. In contrast, Fong and Wang (13) observed that *dnaK* was significantly down-regulated subjected to 6-day desiccation in peanut oil (a_w: 0.52). It is likely that different conditions used to desiccate the cells, as well as different water activities, may contribute to different degrees of *dnaK* expression.

The function of Hsp70 homologue is modified through the interaction with another heat shock protein, GrpE protein, which is encoded by *grpE* gene (50). The realization that in the present study, *grpE* gene was up-regulated in *S.* Typhimurium under desiccations stress led us to hypothesize that heat shock system could play a pivotal role when desiccation-adapted cells are exposed to high temperature. Similarly, Deng et al. (11) also reported the transcription of *grpE* gene in *S.* Enteritidis under desiccation stress in peanut oil.

Genes that were non-differentially expressed under desiccation stress. Two genes, *otsA* and *otsB*, are known to be involved in trehalose biosynthesis, which encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively (51). Finn et al. (12) reported that *otsAB* was up-regulated >11-fold in *S.* Typhimurium under desiccation stress. A recent study by Fong and Wang (13) also saw up-regulations of *otsB* to varying extents in *S.* Typhimurium, Enteritidis, Tennessee, Thompson, and Hartford after a 6-day desiccation in peanut oil. Howells et al. (52) observed that after incubation at 50°C for 7 min, the number of viable cells of a *S.* Typhimurium Δ*otsA* mutant decreased to 0.6% of the initial population, whereas the wild type had an 8% survival rate. Conversely, we did not identify the up-regulation of *otsA* or *otsB* genes previously

reported to be involved in desiccation tolerance of *Salmonella*. Additionally, trehalose was not significantly synthesized during desiccation adaptation ($P>0.05$) (Figure 6.3), which further supports the idea that trehalose synthesis is not essential for the survival of *S.* Typhimurium under desiccation stress in aged broiler litter. In agreement with our gene expression study and trehalose content determination, Δ*otsA* mutant was not impaired in heat resistance as compared to wild type. Our results demonstrated a similarity to those reported by Gruzdev et al. (14), who found that habituation of *Salmonella* to low-a_w environments could not induce the expression of genes involved in trehalose synthesis. An early work by Hengge-Aronis et al. (53) also indicated that *otsAB* mutations did not impair the induction of thermal tolerance in exponentially growing *E. coli* cells. The different results among studies could be explained by the difference in the method used to create a low-a_w condition. Another tentative explanation could be attributed to the fact that different strains were used. In support of this notion, Li et al. (15) found that the trehalose synthesis in *S.* Tennessee was significantly induced by a 2-h desiccation, while no induction was detected in *S.* Typhimurium LT2, indicating that drastic difference in gene expression patterns under desiccation conditions exists among different strains.

The production of curli fimbriae has been reported to aid the persistence of *Salmonella* under desiccation conditions (17). The *Salmonella* strains lacking the ability to produce thin aggregative fimbriae (rdar morphology) could be susceptible to desiccation (16). However, in our study, *agfD* gene responsible for the biosynthesis of thin aggregative fimbriae was not up-regulated during the exposure to desiccation

($P<0.05$), which was further confirmed by the mutation study. Our finding is consistent with some previous studies (12, 14, 15). These observations may indicate that while the formation of filaments may be critical for the long-term persistence in low-a_w environments, this may not be the case when bacteria are desiccated under certain conditions, such as in aged broiler litter. Furthermore, we found that desiccation-adapted rdar morphotype in aged broiler litter did not provide any benefit during heat treatment. Similarly, Scher et al. (21) also observed that the stress response of *S.* Typhimurium pellicle cells (rdar morphotype) to heat at 70°C was not enhanced compared to that of planktonic cells.

During the elongation in fatty acid synthesis, β-hydroxydecanoyl ACP dehydrase (encoded by *fabA* gene) introduces the double bond to the growing fatty acid chain and derives unsaturated fatty acid synthesis from saturated fatty acid synthesis (54). The change in fatty acid composition can affect the fluidity and thermodynamics of cell membrane, which is believed to allow the cell to adapt to the adverse conditions (55). There have been some published reports of the up-regulation of *fadA* gene under desiccation stress. Li et al. (15) reported that *fadA* gene was up-regulated 94- and 64-fold in *S.* Tennessee and *S.* Typhimurium LT2 after desiccation, respectively, which represented the greatest expression change in both strains. When Chen et al. (7) studied the expression of fatty acid biosynthesis-associated genes in *Salmonella* desiccated in granulated sugar (a_w: 0.80) for 14 days, *fadA* gene was observed to be up-regulated. Fong and Wang (13) also reported the significant expression of *fadA* in *Salmonella* following a 6-day desiccation in peanut oil. Conflicting results, however, were obtained in the current

study; no induction of *fadA* gene was detected in *S.* Typhimurium under desiccation conditions in aged broiler litter ($P>0.05$). Similarly, some authors also observed no expression of *fadA* gene in *Salmonella* in response to various desiccation conditions (11, 12, 14). This discrepancy could possibly be accounted for by the different water activities accompanying different methods used to develop a desiccation environment.

In the present study, we observed no induction of *kdpA* gene, encoding a subunit that binds and transports K^+ across the membrane, in desiccation-adapted *S.* Typhimurium. In contrast, in the work of Gruzdev et al. (14), among all desiccation-induced genes in *S.* Typhimurium, the highest up-regulation was observed in *kdpA* gene. However, they noticed that the mutation in *kdpA* gene did not affect the dehydration tolerance in *Salmonella*, but the mutant was significantly compromised during long-term persistence under desiccation conditions. They thus implied that the Kdp high-affinity K^+ uptake system is only involved in the early adaptation of *Salmonella* to the dry environment.

Deng et al. (11) reported that the transcriptions of *cspA*, *sigDE*, and *dps* genes were detected in *S.* Enteritidis in peanut oil. However, none of these genes were up-regulated in our study. With respect to this difference in findings, it can be assumed that a diverse range of stress responses could be induced in *Salmonella* following desiccation, depending on the precise experimental conditions involved. Accordingly, the lack of change in the expression of these genes may be partly due to the less stringent desiccation stress (a_w: 0.81) in aged broiler litter used in our study.

Our results revealed that in response to desiccation in aged broiler litter, *S.* Typhimurium showed a clear shut-off of some biological processes, such as trehalose production, rdar morphology development, and fatty acid synthesis. A conceivable explanation for this is that these processes are very energy-consuming, and a redirection of energy into other more imperative metabolic needs endows *S.* Typhimurium with better survival in low-a_w environments. Likewise, since invasion is not essential for the survival of *S.* Typhimurium under desiccation conditions in aged broiler litter, invasion protein, encoded by *sigDE*, was not synthesized. It can thus be hypothesized that *S.* Typhimurium directs resources away from pathogenicity and into survival in time of desiccation stress.

The effect of desiccation duration on thermal resistance and gene expression. In our study, several time points (3-, 12-, and 24-h desiccation adaptations) were considered. However, we found no significant differences in the thermal resistance or fold changes of *S.* Typhimurium genes among these three time points ($P>0.05$). In contrast, Deng et al. (11) observed that *S.* Enteritidis cells desiccated in peanut oil for 216 h appeared to have increased transcriptional activity compared with cells at 72 and 528 h. More interestingly, we noticed that four up-regulated genes could be induced only within a short period of time (after 1 h but less than 2 h). This finding thus demonstrated that *S.* Typhimurium cells could respond rapidly to the desiccation stress while producing the cross-tolerance to thermal stress, once they are exposed to the conditions in aged broiler litter.

The effect of rehydration on thermal resistance and gene expression. Prior to physical heat treatment, water may be reintroduced into animal wastes via several routes, such as wet cleaning of the processing facility and rainfall events during outdoor storage. Water is also the vector by which bacteria can disseminate in the processing environment (56). In an effort to mimic this scenario of moving from a desiccation to a hydration condition, cells that had been dried in aged broiler litter were rehydrated and then subjected to heat treatment. To our knowledge, this is the first study on the impact of rehydration on the thermal resistance of desiccation-adapted microorganism. It should be noted that after the 3-h rehydration, desiccation-adapted *S.* Typhimurium became significantly less heat-resistant ($P<0.05$). Therefore, the cross-tolerance to heat stress triggered under desiccation stress can be weakened or reversed to normal stage when water is reintroduced into the low-a_w system. Four up-regulated genes became lower expressed ($P<0.05$) or even non-differentially expressed as compared to non-adapted cells ($P>0.05$), suggesting that they appeared to be redundant post-rehydration and our results confirmed their roles in desiccation tolerance. It can thus be speculated that upon desiccation, stress response requirements are greater due to an increased need for some stress-related products. As these genes are not necessary for survival at high a_w, only the expressions of a few essential genes in *Salmonella* maintained active under these conditions. In the study of Finn et al. (12), *otsAB* in *S.* Typhimurium, which had been up-regulated under desiccation stress, returned to basal levels following a 30-min rehydration. Although speculatively, it is possible that the regulations of *dnaK* and *grpE* genes, involved in heat shock protein synthesis, are somehow linked, in a manner as yet

undefined. While *dnaK* gene was up-regulated under desiccation stress, a situation similar to *grpE* gene, their fold changes simultaneously became much lower following rehydration ($P<0.05$).

In our previous study, we developed a two-step heat treatment, consisting of a moist-heat treatment for 1 h at 65°C and a sequential dry-heat treatment for 1 h at 85°C, for rapidly eliminating *S. enterica* in aged broiler litter. Therefore, our current rehydration study has proved that moist heat is a more efficient lethal treatment for microorganisms than dry heat, as *S.* Typhimurium became more heat-sensitive upon rehydration. Our previous study also showed that *S. enterica* desiccation-adapted in aged broiler litter with 20% moisture content (a_w: 0.87) was inactivated more slowly by a two-step heat treatment as compared to 40% (a_w: 0.98) and 50% moisture contents (a_w: 0.99) (8), indicating that desiccation adaptation of *Salmonella* in aged broiler litter with low moisture content could result in enhanced thermal resistance. The findings in the present study have thus proved the significant role of low-a_w condition in aged broiler litter in developing the cross-tolerance in *Salmonella* to thermal stress.

Limitations of this study. We acknowledge that there are some limitations in this study. Due to the lack of mutants in *proV*, *dnaK*, and *grpE* genes, we cannot evaluate the contributions of these three up-regulated genes to the cross-protection in *S.* Typhimurium against heat treatment. While this study provides some information on the response of *Salmonella* cells to desiccation, further characterization to elucidate the significance of these up-regulated systems is warranted so as to gain a more holistic understanding of the processes involved in the cross-protection. Additionally, as only twelve desiccation-

associated genes in *Salmonella* were investigated, a more comprehensive study on the global gene expression using whole transcriptome sequencing technology (RNA-Seq) is necessary.

Conclusions. Our data highlighted the rapid induction of several genes in *S.* Typhimurium, including *rpoS*, *proV*, *dnaK*, and *grpE* genes, that could contribute to the desiccation survival. And *rpoS* gene was identified to be involved in the cross-protection of desiccation-adapted *S.* Typhimurium against high temperature, while trehalose biosynthesis or rdar morphology was not found to play a significant role in this phenomenon. This investigation constitutes the first study on the thermal resistance of previously desiccated microbial cells upon the reintroduction of moisture. Our findings may aid organic fertilizer processors in the design of pathogen control strategies by taking consideration of the cross-protection in order to optimize their thermal processing regimes. Such approaches should be aimed at the elimination of desiccation-adapted human pathogens from animal wastes used as biological soil amendments, thereby improving produce safety and protecting public health.

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Table 6.1 Selected genes and their primers used in qRT-PCR

Gene	Primer sequence (5' to 3')	Primer efficiency (%)	Reference
rpoS (Regulator of the general stress response)	F: CAAGGGGAAATCCGTAAACCC R: GCCAATGGTGCCGAGTATC	100	25
otsA (Trehalose-6-phosphate phosphatase)	F: GGAGTGGCGAGACAGGTAAC R: AGAACCGCATTGGAAAATTG	98	26
otsB (Trehalose-6-phosphate synthase)	F: ACCTTGATGGCACATTGGCAGA R: ACGCCCTGAAATCAATGCCA	96	27
agfD (Positive regulator of thin aggregative fimbriae production)	F: GTGCTCGAGGGACTTCATTAAACATGATG R: GCCGGATCCTGTTTTTCATGCTGTCAC	103	16
kdpA (K^+-transporting ATPase subunit A)	F: GGCGCTACTGACGCTCAATC R: AGGCTTGCCAGTTGGTATTGG	97	28
proV (Osmoprotectant transporters)	F: CCACAATGGTACGCCTTCTCA R: GCATGAGCGCAAATGACTGGA	96	12
fadA (Fatty acid metabolism)	F: ATCTCTCCGCCCACTTAATGCGTA R: AGCCTTGCTCCAGCGTTTGTTGTA	101	15
dnaK (Chaperone protein)	F: CGATTATGGATGGAACGCAGG R: GGCTGACCAACCAGAGTT	104	29
cspA (Cold shock protein)	F: GTTCAACGCTGATAAAGGCTTCGG R: CAGGCTGGTTACGTTGCCAGC	96	30
sigDE (Invasion protein)	F: TGGCATAAAGGGACAGCAC R: AGCGGCAAAGATCGTACAG	99	31
dps (Starvation/stationary phase protection protein)	F: CCCGTAACGATGTATCAGAG R: GCGCTCGGCCATAGTATCCA	103	32
grpE (Heat shock protein)	F: CAGAAAACGCCTGAGGGGCA R: CGCAGGTTTTCCATTTCCGC	96	32
16s rRNA (Reference gene)	F: CCTCAGCACATTGACGTTAC R: TTCCTCCAGATCTCTACGCA	98	33

Table 6.2 Survival of *S.* Typhimurium ATCC 14028 wild type and its mutants in aged broiler litter with 20% moisture content at 75°C

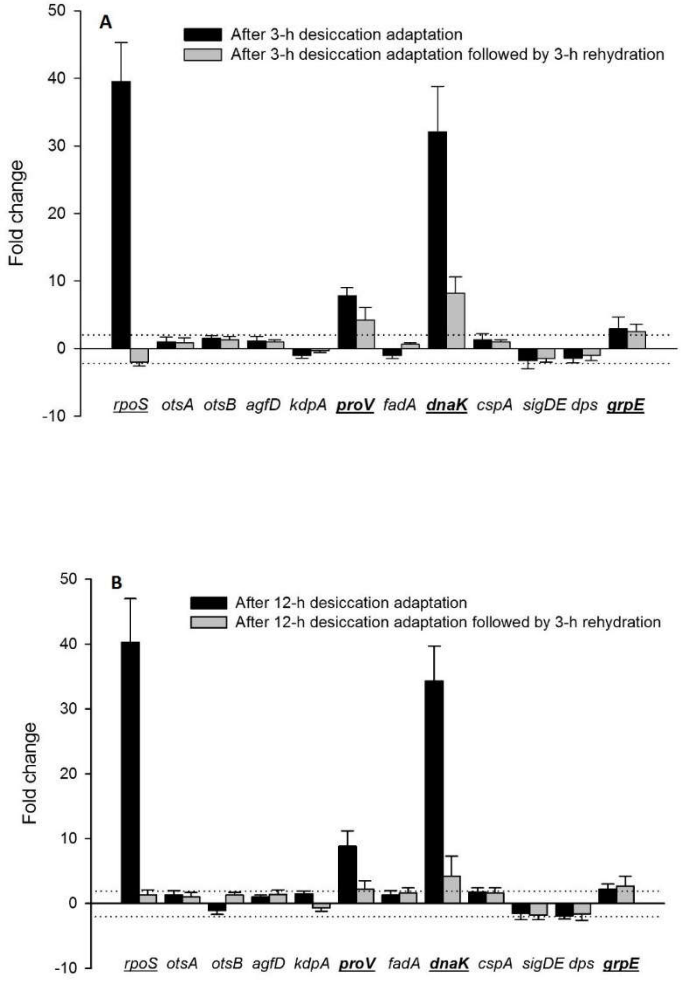
Strain	Desiccation time (h) + rehydration time (h)	Population (log cfu g^{-1}) after (h)				
		0	0.5	1	1.5	2
ATCC	0+0	7.06±0.12A(A)a[a]	2.89±0.13A(B)b	2.14±0.32A(B)b	+[b]	+
14028 (wild	0+3	7.04±0.17(A)a(a)	2.92±0.23(B)b(b)	2.01±0.16(B)b(b)	+	+
type)	3+0	7.00±0.12(A)a	3.92±0.43(A)a	3.82±0.26(A)a	3.74±0.38(A)a	3.53±0.42(A)a
	3+3	7.02±0.01(A)a(a)	3.88±0.21(A)a(a)	2.46±0.11(B)a(a)	2.03±0.14(B)a(a)	1.65±0.22(B)a(a)
	12+0	7.07±0.16(A)a	4.02±0.16(A)a	3.98±0.45(A)a	3.81±0.21(A)a	3.49±0.25(A)a
	12+3	7.07±0.11(A)a(a)	3.71±0.42(A)a(a)	2.57±0.27(B)a(a)	1.93±0.26(B)a(a)	1.74±0.12(B)a(a)
	24+0	7.09±0.08A(A)a	3.88±0.33A(A)a	3.92±0.18A(A)a	3.80±0.58A(A)a	3.58±0.42A(A)a
	24+3	7.00±0.12(A)a(a)	3.83±0.24(A)a(a)	2.49±0.31(B)a(a)	2.13±0.19(B)a(a)	1.60±0.32(B)a(a)
IB43	0+0	7.04±0.12Aa	2.55±0.20Bc	-[c]	-	-
(Δ*rpoS*)	24+0	7.00±0.00Aa	2.59±0.19Bc	-	-	-
XF373	0+0	7.03±0.01Aa	2.91±0.21Ab	1.96±0.15Ab	+	+
(Δ*otsA*)	24+0	7.03±0.06Aa	3.99±0.01Aa	3.96±0.15Aa	3.83±0.43A	3.60±0.14A
MAE 110	0+0	7.06±0.05Aa	2.90±0.25Aa	2.09±0.15Aa	+	+
(Δ*PagfD*)	24+0	7.03±0.11Aa	4.05±0.27Ab	3.95±0.33Ab	3.77±0.13A	3.54±0.59A
MAE 119	0+0	7.05±0.15Aa	2.85±0.22Aa	2.13±0.25Aa	+	+
(Δ*agfD*)	24+0	7.04±0.06Aa	4.08±0.43Ab	3.90±0.32Ab	3.81±0.09A	3.59±0.39A

[a]Data are expressed as mean±SD. According to the LSD test, in the same column, means with different uppercase letters for the same desiccation adaptation time were significantly different ($P<0.05$), while means with different lowercase letters for the same strain were significantly different ($P<0.05$). For wild type in the same column, means with different uppercase letters in parentheses with the same desiccation adaptation time are significantly different ($P<0.05$), while means with different lowercase letters in parentheses with different desiccation adaptation times after rehydration were significantly different ($P<0.05$).

[b]+, detectable only by enrichment.

[c]-, not detectable by enrichment.

Figure 6.1 Fold changes of *S.* Typhimurium wild type genes after 3- (A), 12- (B), and 24-h (C) desiccation adaptations and after 3-h rehydration. Differentially expressed genes were defined as significantly up- or down-regulated ≥2 folds (horizontal dotted line) between desiccation-adapted or rehydrated cells and control ($P<0.05$). Genes underlined but not in bold were significantly up-regulated ($P<0.05$) after desiccation adaptation but non-regulated after rehydration, while genes underlined and in bold were significantly up-regulated ($P<0.05$) both after desiccation adaptation and after rehydration.



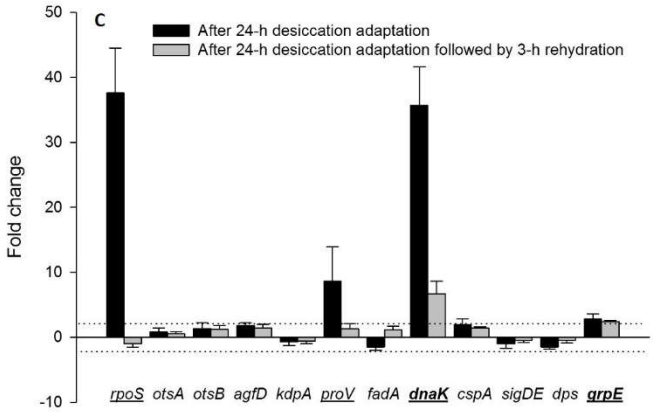

C
After 24-h desiccation adaptation
After 24-h desiccation adaptation followed by 3-h rehydration
Fold change
50
40
30
20
10
0
-10
rpoS otsA otsB agfD kdpA proV fadA dnaK cspA sigDE dps grpE

Figure 6.2 Fold changes of differentially expressed *S.* Typhimurium wild type genes after 1- and 2-h desiccation adaptations. Differentially expressed genes were defined as significantly up- or down-regulated ≥2 folds (horizontal dotted line) between desiccation-adapted cells and control ($P<0.05$).

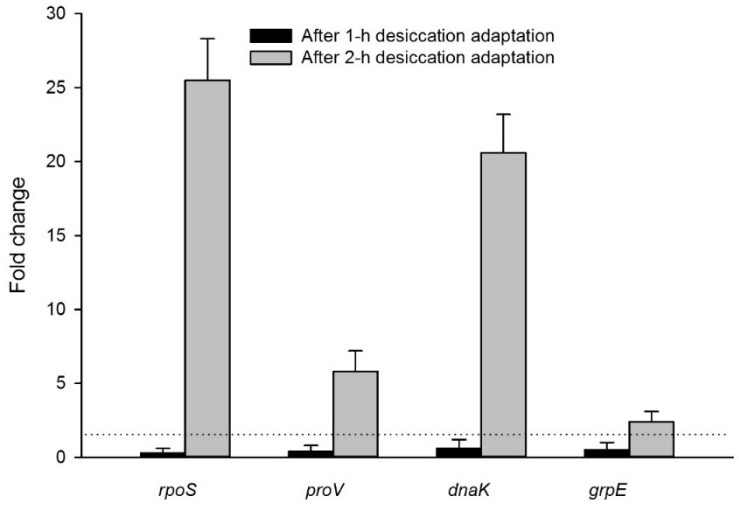
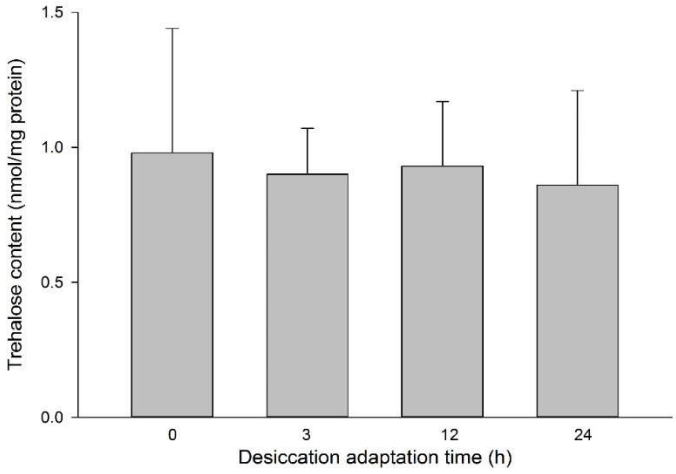


Figure 6.3 Trehalose contents in *S.* Typhimurium wild type after 3-, 12-, and 24-h desiccation adaptations.



CONCLUSIONS

Poultry litter is usually recycled into the agricultural land as a biological soil amendment. Poultry litter may possibly contain a variety of pathogens that can pose a risk to humans who consume the contaminated produce. The composting process can kill pathogens and create a biological soil amendment beneficial for agricultural land application. Some pathogens may have the potential to survive and persist for extended periods of time in raw poultry litter or its inadequately composted products. A small population of cells may even regrow to high levels when the conditions are favorable. Subsequent thermal processing can be used to inactivate pathogens in poultry litter prior to land application. However, some microbial populations may become adapted to the stressful environment during composting or stockpiling and develop cross-protection against subsequent thermal stress.

Our results demonstrated that desiccation-adapted *Salmonella* cells could survive much longer as compared to non-adapted cells during thermal processing of poultry litter. And the thermal resistance of desiccation-adapted *Salmonella* in poultry litter could be affected by storage time and ammonia content of poultry litter. A two-step thermal processing technique (a 1-h moist-heat treatment at 65°C and 100% RH followed by a 1-h dry-heat treatment at 85°C) was developed to rapidly inactivate desiccation-adapted *Salmonella* in poultry litter. To validate the thermal processing of poultry litter in industry settings, suitable indicator microorganisms with similar survival behaviors to *Salmonella* are needed. We found that indigenous enterococci could be considered as an indicator microorganism, as our data demonstrated that indigenous enterococci showed a

better prediction of the survival behavior of desiccation-adapted *Salmonella* during heat treatment. To explore the underlying mechanisms of the enhanced heat resistance of desiccation-adapted *Salmonella*, based on the qRT-PCR and mutation study, *rpoS* gene was identified to be involved in the cross-protection of desiccation-adapted *Salmonella* against high temperature. This study validated the effectiveness of thermal processing being used for producing poultry litter free of *Salmonella* contamination. Our findings may aid organic fertilizer processors in the design of pathogen control strategies by considering the cross-protection in order to optimize their thermal processing regimes. Future studies should be conducted to validate the time-temperature combinations of thermal processing of poultry litter under various conditions in industry settings. Moreover, it is also warranted to investigate the microbial community profiles of poultry litter prior to and after thermal processing, as well as to study the influence of high temperatures on beneficial microorganisms which can enhance plant productivity and yield in cropping systems.